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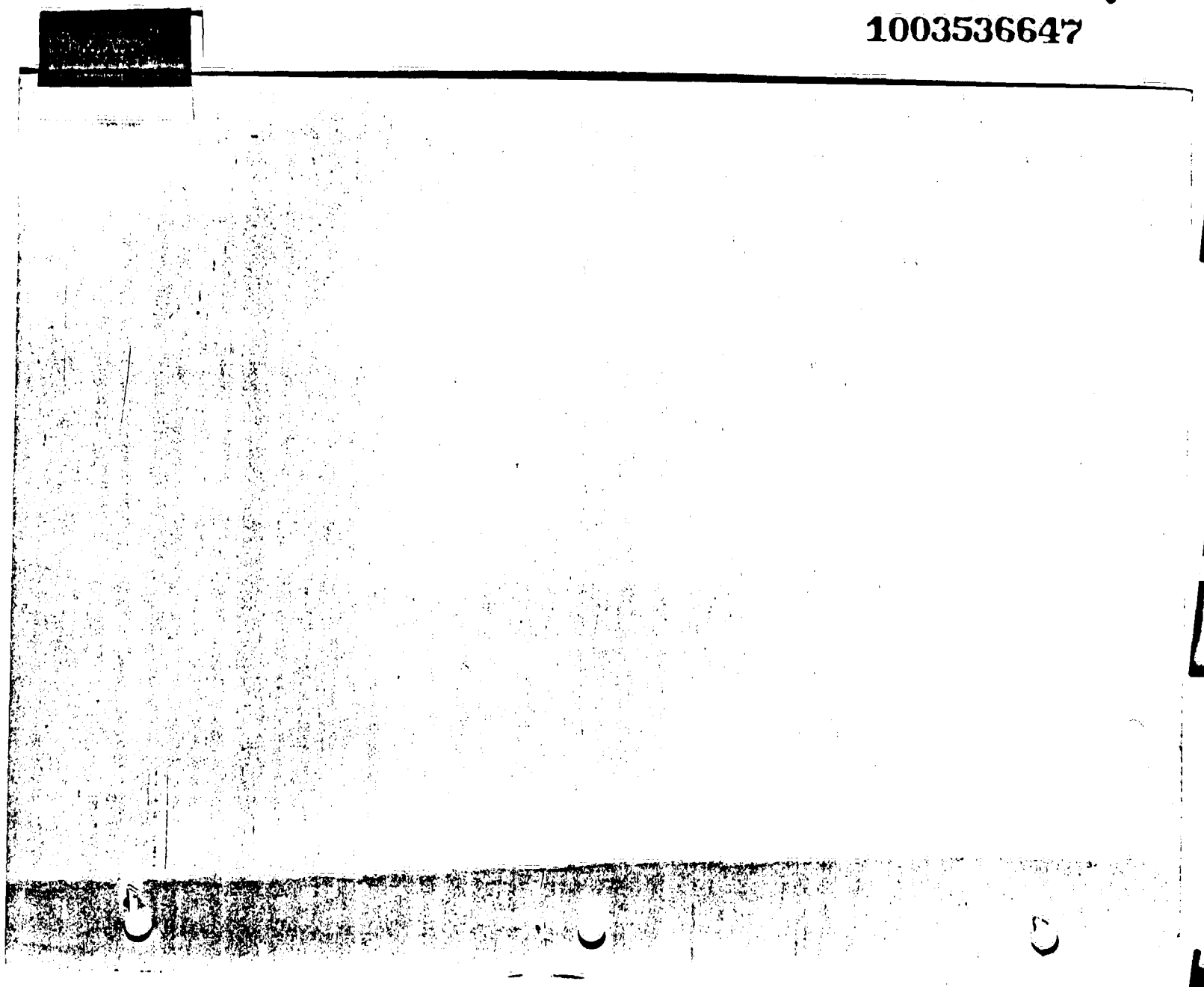

Smoke Facility
and Inhalation

SMOKE INHALATION CARCINOGENESIS STUDIES IN MICE

CTR Contract #22
MA Contract 2224

CONTRACT RENEWAL PROPOSAL
(SECOND YEAR)
FOR THE PERIOD
July 1, 1975 - June 30, 1976

February 12, 1975

Vincent L. Ruwet
Vice President, Contracts and Administration

TO: The Council for Tobacco Research U.S.A.
110 East 59th Street
New York, N.Y. 10022

FROM: Microbiological Associates
A Division of Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

Prepared by:

Carrie E. Whitmire, Ph.D.
Project Director

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I Progress Report

Since this contract was initiated July 1, 1974 an architectural plan has been drawn, the building contract let and rearrangement of the facilities are well underway. We anticipate the building to be ready for occupancy by June 1, 1975. This will allow a month to prepare the facility for work by July 1, 1975 in the new contract year.

The equipment has been ordered and much of it has begun to arrive. The only item which has given us considerable problems has been the X-ray machine. We have not been able to achieve the desired resolution to provide for sensitive lung cancer diagnosis to date. There are several more approaches to be taken which may allow us to obtain this equipment in the new contract year.

The various studies to define the lung carcinogenesis bioassay system with BaP have been quite encouraging and have been reported on in the progress report for CTR-14.

The dosimetry studies with Oak Ridge National Laboratories has been initiated. A group of studies have been designed and are included in this proposal.

II Proposal for New Contract Year

A. Smoke Studies

These were outlined in last year's proposal and will be more closely defined before initiated on occupation of the new facilities. At this time various experiments are still in progress which could influence the final experimental design.

B. Dosimetry Studies

Studies have been designed in collaboration with Dr. James Stockely at ORNL and are outlined on the following pages. These studies will be carried out on the Walton Smoking Machine. These data will be used to design dosimetry studies on the new smoking machine to be used in the inhalation studies.

PROTOCOL FOR MOUSE DOSIMETRY EXPERIMENTS ON WALTON HORIZONTAL SMOKING MACHINE

Prepared by

C.E. Whitmire
Microbiological Associates

and

J.R. Stokely
Oak Ridge National Laboratory

These protocols outline proposed collaborative studies by Microbiological Associates and Oak Ridge National Laboratory to investigate mouse dosimetry on the Walton Horizontal Smoking Machine. The objectives of these studies are:

1. To define the dose of tobacco smoke particulates received by mice under selected exposure conditions (exposure time and smoke concentration),

2. To ascertain possible effects of sex and strain on dosimetry so that a rational selection can be made for future studies of biological impact,

3. To determine retention and clearance rate of smoke particulates after exposure, and

4. To determine cumulative dose and long-term retention of smoke particulates under typical exposure regimes.

Responsibilities of Oak Ridge National Laboratory are the following:

1. Weight and RTD selection of cigarettes.
2. Labeling of cigarettes with ^{14}C tracers.
3. Quality control of cigarettes (determination of nicotine, TPM, tar and ^{14}C delivery of selected radiolabeled cigarettes).
4. Shipping of radiolabeled cigarettes to Microbiological Associates.
5. Construction and testing of apparatus for sampling exposure chamber.
6. Instruction and assistance on operation of smoking machines, sampling apparatus, and cigarette conditioning and handling.
7. Analysis of animal tissues for ^{14}C tracers.
8. Analysis of input Cambridge filter pads for nicotine and ^{14}C tracer and chamber samples for ^{14}C tracer.
9. Calculations to obtain the following results:
   a. absolute activity (dpm) of tracer in each tissue specimen.
   b. absolute tar (mg) deposited in each tissue specimen based on tracer deposition.
   c. percent distribution of activity among various tissue specimens for each animal.
   d. dose expressed as percentage of smoke input to exposure chamber.

Responsibilities of Microbiological Associates are the following:

1. Calibration of smoking machines (puff volume, puff time, exposure time, purge time).
2. Conditioning and weighing of labeled cigarettes after receipt from Oak Ridge National Laboratory.
3. Collection of samples of smoke input to smoking machines and chamber samples obtained during animal exposures.
4. Animal conditioning.
5. Animal exposure.
6. Animal sacrifice and dissection.
7. Shipping of tissue specimens, input filter pads and chamber samples to Oak Ridge National Laboratory.

Experiment I. Effect of Exposure Time

1. One mouse strain: C3H/Anf Cum
2. One sex: female
3. Four exposure time intervals: 10,20,30,40 seconds
4. One exposure concentration: 10%
5. Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus
6. Number of mice per exposure: 10(plus 10 scrubs)
7. Number of cigarettes per exposure: 3 (2 for chamber input, 1 for exposure)
8. Chamber samples per exposure: 1
9. Cigarette type: Kentucky Reference 1A) loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested ($\pm$ 20 mg, $\pm$ 5 mm H_2O)
10. Number of repetitive exposures: 4 (40 Mice)
11. Instant sacrifice
12. Tissue specimens: 640
13. Total cigarettes: 60 (assume 25% loss).

Experiment II. Effect of Smoke Concentration

1. One exposure time interval: based on experiment I
2. Five exposure concentrations: 20% (2 cigarettes-384 ml chamber),
   30% (3 cigarettes-384 ml chamber),
   5% (1 cigarette-768 ml chamber),
   10% (2 cigarettes-768 ml chamber),
   15% (3 cigarettes-768 ml chamber)
3. Number of cigarettes per exposure: 6(20%), 9(30%), 3(5%), 6(10%), 9(15%)
4. One mouse strain: C3H/Anf Cum
5. One sex: female
6. Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus
7. Number of mice per exposure: 10 (plus 10 scrubs)
8. Chamber samples per exposure: 1
9. Cigarette type: Kentucky Reference 1A1 loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested ($\pm$ 20 mg, $\pm$ 5 mm H_2O)
10. Number of repetitive exposures: 4 (40 mice)
11. Instant sacrifice
12. Total tissue specimens: 800
13. Total cigarettes: 165 (assume 25% loss)

Experiment III. Effect of Sex

1. Two sex: male and female
2. One exposure time interval: based on experiment I
3. One exposure concentration: based on experiment II
4. Number of mice per exposure: 20 (10 male, 10 female)
5. Number of repetitive exposures: 4 (40 mice)
6. Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus
7. Chamber samples per exposure: 1
8. Cigarette type: Kentucky Reference 1A1 loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested ($\pm$ 20 mg, $\pm$ 5 mm H_2O)
9. Instant sacrifice
10. Tissue specimens: 300
11. Total cigarettes: 15 (assume 25% loss).

Experiment IV: Strain Differences

1. Four mouse strains: C3H/f, C57BL/6, DBA/2, B6C3F1
2. Sex: based on experiment III (male, female, both)
3. One exposure time: based on experiment I
4. One exposure concentration: based on experiment II
5. Four tissue samples: skinned head, upper trachea and larynx, lungs and lower half of trachea, stomach and esophagus
6. Number of mice per exposure: 20 (10 each of 2 strains or sexes
7. Number of cigarettes per exposure: 3 (2 for chamber input, 1 for exposure)
8. Chamber samples per exposure: 1
9. Cigarette type: Kentucky Reference 1A1 loaded with 5×10^6 dpm ^{14}C-dotriacontane--weight and RTD tested (±20 mg, ± 5 mm H_2O)
10. Instant sacrifice
11. Tissue specimens: 640 (1 sex)
    1280 (2 sexes)
12. Number of cigarettes: 30 (1 sex)
    60 (2 sexes) (assume 25% loss)

Experiment V: Retention Period of ^{14}C-Dotriacontane

1. One mouse strain: C3H/f
2. One sex: based on experiments III and IV
3. One exposure time: based on experiment I
4. One exposure concentration: based on experiment II
5. Five tissue samples per animal: same as experiment I plus composite sample of all other animal tissues--animal skinned--skin not included in composite.
6. Number of mice per exposure: 20
7. Five sacrifice times after smoking: 0.25, 1, 4, 16, 24 hours. Four animals sacrificed at each time.
8. Number of cigarettes per exposure: 3
9. Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8 dpm) ^{14}C-ditriacontane.
10. Number of repetitive exposures: 4
11. Tissue specimens: 400
12. Number of cigarettes: 15 (assume 25% loss)

Experiment VI: Retention Period of ^{14}C-Benz(a)pyrene

1. Cigarette type: 1A1 loaded with maximum amount (up to 1 x 10^8 dpm) ^{14}C-benz(a)pyrene
2. One mouse strain: C3H/f or B6C3F1
3. One sex: based on experiments III and IV
4. One exposure time: based on experiment I
5. One exposure concentration: based on experiment II
6. Five tissue samples per animal: same as experiment I plus composite sample of all other animal tissues--animal skinned--skin not included in composite.
7. Number of mice per exposure: 20
8. Five sacrifice times after smoking: 0.25, 1, 4, 16, 24 hours. Four animals sacrificed at each time.
9. Number of cigarettes per exposure: 3
10. Cigarette type: 1A1 loaded with maximum amount (up to 1 x 10^8 dpm) ^{14}C-ditriacontane.
11. Number of repetitive exposures: 4
12. Tissue specimens: 400
13. Number of cigarettes: 15 (assume 25% loss)

Experiment VII: Retention Period of ^{14}C-Nicotine

1. Five sacrifice times after smoking: immediately, 0.25, 0.5, 1, 4 hours after smoking. Four animals sacrificed at each time.
2. Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8) ^{14}C-nicotine.
3. One mouse strain: C3H/f
4. One sex: based on experiments III and IV
5. One exposure time: based on experiment I
6. One exposure concentration: based on experiment II
7. Five tissue samples per animal: same as experiment I plus composite sample of all other animal tissues--animal skinned--skin not included in composite.
8. Number of mice per exposure: 20
9. Five sacrifice times after smoking: 0.25, 1, 4, 16, 24 hours. Four animals sacrificed at each time.
10. Number of cigarettes per exposure: 3
11. Cigarette type: 1A1 loaded with maximum amount (up to 1×10^8 dpm) ^{14}C-dotriacontane.
12. Number of repetitive exposures: 4
13. Number of cigarettes: 15 (assume 25% loss).

Experiment VIII: Comparative Retention Periods for Two Mouse Strains

1. Two strains: based on experiment IV
2. One sex: based on experiments III and IV
3. One exposure time: based on experiment I
4. One exposure concentration: based on experiment II
5. Two retention periods: based on experiments V, VI, and VII
6. Five tissue specimens per animal: experiment V
7. Twenty mice per exposure: ten mice sacrificed at each retention period
8. Number of repetitive exposures: 2
9. Three type cigarettes: Kentucky Reference 1A1 loaded with ^{14}C-nicotine, ^{14}C-benz(a)pyrene, or ^{14}C-dotriacontane (maximum load)
10. Tissue specimens: 1200
11. Number of cigarettes: 15(assume 25% loss).

C3V
DBA

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Experiment VIII: Comparative Retention between the two

Experiment IX: Cumulative Dose and Long Term Retention

1. Number of radiolabeled cigarettes given per 8 hour day: 2, 5, 10, 20
2. One strain - based on experiment VIII
3. Four animals sacrificed at each of the following times after exposure: immediately, 4, 24, 48, 120 hours
4. Three type cigarettes: Kentucky Reference 1A1 loaded with ^{14}C-nicotine, ^{14}C-benz(a)pyrene, or ^{14}C-dotriacontane (maximum load) or all three at one time.
5. Number of repetitive tests: 1
6. Other conditions: same as experiment VIII
7. Tissue specimens: 1200
8. Number of cigarettes: 169 (assume 25% loss).

Summary of Tissue Specimens and Number of Cigarettes

Experiment	Tissue Specimens[1]	Number of Cigarettes[2]
I	640	60
II	800	165
III	320	15
IV	1280	60
V	400	15
VI	400	15
VII	400	15
VIII	1200	15
IX	1200	169
	6640	529

[1] Maximum

[2] Assume 25% loss

III Budget

The budget has been divided into the various support and scientific sections required to carry out the proposed studies. Several items of equipment have been included in this year's budget which will be needed to carry out the program.

BUDGET CTR-22 (July 1, 1975 - June 30, 1976)

		Section A*	B	C	D	E	Totals
A.	Direct Labor (Schedule A)	$ 23,240	$ 20,234	$ 29,573	$ 54,509	$110,645	$238,201
B.	Overhead (115% of A)	26,726	23,269	34,009	62,685	127,242	273,931
C.	Other Direct Costs	--	7,000	35,000	10,000	45,000	97,000
D.	Travel	2,000	--	--	1,500	--	3,500
E.	Total (A-D)	51,966	50,503	98,582	128,694	282,887	612,632
F.	G & A (16% of E)	8,315	8,080	15,773	20,591	45,262	98,021
G.	Total	60,281	58,583	114,355	149,285	328,149	710,653
H.	Fixed Fee	6,698	6,509	12,705	16,586	36,457	78,955
I.	Total Before Equipment and Overtime Premium	66,979	65,092	127,060	165,871	364,606	789,608
J.	Equipment	1,500	300	8,000	5,500	10,000	25,300
K.	Overtime Premium	--	--	--	1,500	--	1,500
L.	Total	$ 68,479	$ 65,392	$135,060	$172,871	$374,606	$816,408
M.	Contingency						83,592
N.	Total Cost						$900,000

*Sections: A. Administration
B. Data Processing
C. Dosimetry with ORNL
D. Histopathology
E. Inhalation Studies

SCHEDULE A - Direct Labor For CTR Contract 22
(July 1, 1975 - June 30, 1976)

	Section/Personnel	$/Hr.	% Time	Hours	$
A.	Administration				
	C.E. Whitmire, Ph.D.	14.38	50	963	$13,848
	C.F. Demoise, Ph.D.	8.18	25	482	3,943
	Vacancy, Adm. Assist.	4.08	50	963	3,929
				2,408	$21,720
			7% Merit Raise		1,520
					$23,240
B.	Data Processing				
	M. Haven, M.S.	10.58	50	963	$10,189
	P. Gradwell, Data Tech.	3.84	30	578	2,220
	Vacancy, Data Tech.	3.50	50	963	3,371
	B. Ross, Key Punch Tech.	3.25	50	963	3,130
				3,467	$18,910
			7% Merit Raise		1,324
					$20,234
C.	Dosimetry Studies with ORNL				
	D. Dansie, Tech.	3.80	100	1,926	$ 7,319
	Vacancy, Tech.	4.20	100	1,926	8,089
	G. Gomez, Jr. Tech.	3.25	100	1,926	6,259
	J. Fernandez, Anim. Care.	3.10	100	1,926	5,971
				7,704	27,638
			7% Merit Raise		1,935
					$29,573
D.	Histopathology				
	Vacancy, D.V.M.	17.30	100	1,926	$33,320
	S.A. Gosnell, Super. Tech.	4.30	50	963	4,141
	Vacancy, Tech.	3.50	100	1,926	6,741
	Vacancy, Tech.	3.50	100	1,926	6,741
				6,741	$50,943
			7% Merit Raise		3,566
					$54,509
E.	Inhalation Studies				
	7 Vacancies, Jr. Tech.	3.50	100	13,482	$47,187
	4 Vacancies, Jr. Tech.	3.50	50	3,852	13,482
	A. Saborit, Washroom Aide	3.39	100	1,926	6,529
	2 Vacancies, Washroom Aide	3.20	100	3,852	12,326
	1 Vacancy, Anim. Care.	3.35	100	1,926	6,452
	A. Lopez, Sr. Tech.	4.80	100	1,926	9,245
	G. Griffin, Tech.	4.25	100	1,926	8,186
				28,890	$103,407
			7% Merit Raise		7,238
					$110,645
Totals			25.5 Man Years	49,210	$238,201

Schedule B: CTR-22 (July 1, 1975 - June 30, 1975)

Desks, files	$ 450
Typewriter	650
2 calculators	400
Computer files	300
Gas chromatographic and other dosimetry monitoring equipment	8,000
Pathologist's microscope tables, etc.	1,500
Microprojector	4,000
X-ray machine and safety shield	10,000
	$25,300

Basic and
Supportive Research

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

March 4, 1975

To: The Scientific Advisory Board

Subject: Basic and Supportive Contract No. 14B - Microbiological Associates, July 1, 1975 - June 30, 1976

The studies to determine susceptibility to lung carcinogenesis in genetically defined mouse strains have been carried out under the direction of Drs. Carrie Whitmire and Richard Kouri at Microbiological Associates.

The activities to be undertaken during each funding period are proposed by the contractor with close staff liaison. The Scientific Director and Research Director further refine these proposals, deleting or adding as felt appropriate. Site visits are arranged between contractor, SAB members of the contract committee, CTR representatives including Scientific Director and Research Director, and, as of the forthcoming period of Contract No. 14B, outside consultants with special scientific expertise in the area under consideration. (Periodic site visits are made during the year to follow up the progress). Recommendations and proposals are then submitted to the SAB for formal considerations. Final budgetary negotiations are carried out by CTR's Scientific Director and contractor following the SAB meeting and in line with budgetary recommendations, limitations, etc. of the CTR.

The current proposal, subject to negotiations, revision pending, recommendations of reviewing groups, etc. is attached. This represents a consolidation of Grants No. 2 and 14, arbitrarily divided some years ago for budgetary considerations.

Funding history for the past year:

		Amount	Annual Amount
No. 2F	2/1/74 - 1/31/75	$137,500	
No. 14A	7/1/74 - 6/30/75	$350,000	
			$487,500
No. 14AS	Supplement 1/31/75 - 6/30/75 (to bring two contracts to same initiation date).	$ 58,000	

Requested (subject to SAB approval, negotiation, etc.)

No. 14B	7/1/75 - 6/30/76	$522,794

J.H.K.

10035366 72

DEVELOPMENT OF A MOUSE MODEL SYSTEM FOR GENETIC SUSCEPTIBILITY AND ITS RELATIONSHIP TO IN VIVO LUNG CARCINOGENESIS

CTR Contract #114
MA Contract 2222

CONTRACT RENEWAL PROPOSAL
FOR THE PERIOD
July 1, 1975 - June 30, 1976

February 12, 1975

Vincent L. Ruwet
Vice President, Contracts and Administration

To: The Council for Tobacco Research U.S.A.
110 East 59th Street
New York, N.Y. 10022

From: Microbiological Associates
A Division of Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

DEVELOPMENT OF A MOUSE MODEL SYSTEM FOR GENETIC SUSCEPTIBILITY AND ITS RELATIONSHIP TO IN VIVO LUNG CARCINOGENESIS

It has been long evident that genetic susceptibility is an inherent factor in the incidence of many types of cancer. The human familial incidences of breast and colonic cancer, the predominance of leukemia in a segregant population, and other similar analogies make this a reasonable hypothesis.

Inbred mouse chemical carcinogenesis models, dependent upon subcutaneous chemical carcinogenesis and more recently intratracheally introduced chemical carcinogenesis studies have reinforced the human genetic susceptibility data.

The current "Mouse Model System" contract has investigated and developed the mouse system to four ends. First, to define the appropriate strains of mice to investigate cigarette smoke inhalation effects. Secondly, the program is designed to further define susceptibility to lung cancer. Thirdly, the models developed will be used to attempt to define in a susceptible animal the element risk from exposure to pure chemicals found in tobacco smoke given at different doses. Lastly, long term immunocompetency studies following treatment with chemical carcinogens in susceptible strains have been undertaken in collaboration with Scripps. The techniques developed in these studies will be integrated in a study to define the effects of chronic cigarette smoke exposure and immunocompetency.

Prepared by:

Carrie E. Whitmire, Ph.D.
Co-Project Director

Richard E. Kouri, Ph.D.
Co-Project Director

Charles F. Demoise, Ph.D.
Associate Project Director

Bernard Sass, D.V.M.
Pathologist

Miles J. Haven, M.S.
Scientific Data Processing
and Analysis Manager

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SUMMARY PROGRESS REPORT

I. SUMMARY PROGRESS REPORT

This report covers all research projects undertaken at MA for CTR on Contracts 2, 14 and 22 during 1974. Although funding has been under several contracts we have undertaken integrated research in an effort to answer some of the biological mechanisms involved in the initiation and development of cancer. In order to study these mechanisms we have studied various genotypically different mouse strains best suited to a particular mechanism.

The CTR-MA contracts have two basic functions:
First, to define a mouse model system for studying various aspects of carcinogenesis.

Second, to undertake screening of known carcinogens, cigarette smoke condensates and condensate fractions by subcutaneous and pulmonary inoculations of mice.

A. Model Systems

1. Chemical Carcinogenesis

a. SC Route (Trioctanoin Vehicle)

In order to define the mouse model systems, we have undertaken subcutaneous carcinogenesis studies with MCA to determine the relative sensitivity of several mouse strains as quickly as possible, and to compare subcutaneous and intratracheal susceptibility. This MCA-SC study (CTR-4) has been completed. Based on the MCA dose required to produce fifty percent tumors (TuD_{50}) in eight months, the most susceptible strain was the C3H/f, followed by the B6C3F1 hybrid using the C3H/f Mai and the C57BL/6 Cum mice. This hybrid is not available commercially but should be considered as a possible susceptible strain after it is characterized for other carcinogens. The time required for tumor development more closely paralleled that of the C3H parent. All the strains were AHH inducible. The C57BL and C57BL/6 mice were virtually negative for type C RNA gs antigen, while nearly all the C3H/f mice were gs^+. The hybrid mice appear to take on the same gs antigen expression as the C3H/f parent.

b. SC Route (Beeswax:Trioctanoin (B:T) Vehicle)

In order to better understand the relationship of tumor induction with B:T vehicle in subcutaneous tumor induction as used for assay of carcinogenic potential of CSC materials, CTR-19 was undertaken with MCA, BaP and DMBA in C57BL/6, C3H/f, DBA/2 and BC3F1 mice. Although these studies are still in progress it would appear that the use of B:T as a vehicle reduces the tumorigenic potential 16x.. This may represent a difference

in tumor latency, therefore the final results will be necessary before we will see the effect.

c. MCA-IT Route

We undertook two initial studies an in effort to repeat and expand on Nettesheim's intratracheal administration of MCA and the subsequent development of squamous cell carcinomas (SCC) (CTR-3 and 5). Both studies produced only limited numbers of SCC probably due to the use of sonicated material. Later studies with unsonicated material indicate that the particle size of the carcinogen is significant, not only in the type of tumor which develops, but also the latency period for tumor development is greatly reduced with larger particulate material.

Although few SCC were produced in CTR-3 and 5, significant findings were made in CTR-3. It would appear that the hybrid BC3F1 is more susceptible to lung tumor induction than the C57BL, C57BL/6, or C3H/f mouse. This will be studied in later studies. The histological diagnosis of these tumors were adenocarcinomas and alveolar adenomas with relatively few squamous cell carcinomas. In CTR-5 the genetic determination of AHH inducibility was confirmed and it was found that only those mice which were AHH+ developed tumors in the backcross animals (B6 x B6D2). Both CTR-3 and 5 demonstrated that 500µg MCA is exceedingly toxic and many animals are lost during the treatment period due to pneumonia.

Additional studies were undertaken to study the inoculation schedule and diluent for suspending the MCA (CTR-3A and B). These studies have not been completed, however, it would appear that early tumors, and SCC in particular, do not develop with trioctanoin as a diluent. Mice were also injected on a weekly vs. a biweekly schedule. It would appear that the biweekly schedule may be preferable and gel the better vehicle.

Other studies using the IT procedure with MCA are in progress in an effort to determine the TuD_{50} dose for MCA (CTR-7, 7A and 7B). These studies have not been completed, however, they do demonstrate that the survival rate is much better with 250, 125 and 62.5µg MCA and that tumors do occur with a longer latency period with 125 and 62.5µg MCA, particularly when given 12x and to a lesser degree with 6x.

The IT route of MCA is also being used to study the genetic cross and backcross of C3H/f and DBA/2 (CTR-39). Although these studies are still in progress, early results would indicate that tumors have occured in the AHH+ animals.

d. BaP-IT route

Two studies are in progress to determine if Saffiotti's procedure of combining BaP and Fe_2O_3 would result in the production of SCC in mice. The C3H/f mouse has been given 3 dose levels of BaP (0.6, 1.2, 1.8mg/injection) without Fe_2O_3 or combined with 0.6mg Fe_2O_3. These combinations have been administered 5 and 10 times IT in CTR-43. In CTR-44 the DBA/2 mouse

has been given only the highest dose of BaP (1.8mg) with and without 0.6mg Fe_2O_3. The DBA/2 mice have been on test for only 21 weeks and have shown no evidence of lung involvement. The C3H/f mice have been on test for 38 weeks and up to 75% of the animals treated 10 times with BaP and Fe_2O_3 have developed squamous lesions and/or squamous cell carcinomas. It appears that mice treated 5x may develop tumors, however the latency period is longer and the incidence will be lower.

e. DEN-IT Route DENA ?

In CTR-18 DEN was given to DBA/2 mice in the hopes of developing SCC, however, very few lung tumors developed, however these animals developed liver carcinomas. This model opens up another important aspect of the IT route in that it demonstrates the respiratory route of chemical carcinogens for the induction of tumors in other parts of the body.

f. Beeswax:Trioctanoin Lung Pellet Carcinogenesis

The dose of carcinogen which can be administered by IT inoculation is of necessity rather small and is subject to the need for multiple injections under anesthetic. The implantation of wax pellets of carcinogen used by Stanton in rats has been adapted to mice in our laboratory. This procedure allows larger doses to be given at one time and, in theory, should require only one injection. There are difficulties with this procedure, however, which were not realized when these studies were undertaken. There is a great difference in the leaching rates of different chemicals. There is of course differences in leaching related to the ratios of beeswax to trioctanoin and to carcinogens which are liquids. There are differences in the leaching rate based on the size of the pellet and on the tissue in which it is situated since carcinogens may be water soluble or fat soluble. In the case of multicomponent materials, as (CSC,) the leaching rate of each component will be different thus instead of having a constant exposure to all carcinogens there is probably a serial exposure to one or more carcinogens at a time.

Several studies are now underway using the lung wax implantation procedure. The nitrosamines are being studied this way since it appeared a safer way of handling these materials than by IT inoculation. DMN has been given to both AHH inducible and non-inducible mice in an effort to determine if AHH induction can be correlated with dimethylase activity in any way. This has been done in CTR-18A.

Since DEN, DBN, PYR and PIP have been shown to produce varying lung pathology in C57BL/6 mice when given IP to newborn mice it was thought the aspects of lung carcinogenesis should be pursued by use of the lung pellet procedure. This has been initiated in CTR-45.

To further evaluate this procedure we have also put on test two dose levels of MCA, BaP, DEN and DMBA in C3H/f, C57BL/6 and DBA/2 mice in CTR-18B.

2. AHH Inducers

Studies were undertaken using a chemical (TCDD) which induces AHH regardless of genotype (CTR-15, 16, 17). Considerable toxicity was encountered. Both C57BL/6 and DBA/2 mice were tested to determine the effects of TCDD on subcutaneous tumor induction. The results with the TCDD are compatible with the idea that AHH induction (via TCDD) simultaneous with MCA treatment yields more tumors than MCA alone, but the results with dioxane are difficult to assess. Why a 48 hours pretreatment with 0.010ml dioxane should enhance MCA-induced tumorigenesis cannot be explained at this time. The fact that both the low and high TCDD levels, when given 48 hours before MCA, had no effect, yet dioxane was also in these treatments, indicates that whatever the effect of dioxane, it is cancelled, if TCDD is present.

This study has been repeated in DBA/2 mice in an effort to determine the problems with dioxane. Results to date (22nd week) indicate that: a) 3% tumors have been observed in MCA-treated D2 mice, b) about 5% tumors in MCA plus dioxane treated mice and MCA plus TCDD, when given IP, treated mice, and c) 31% tumors when TCDD plus MCA given in same vehicle simultaneously.

3. Immunocompetence

The effects of chemical carcinogens on the immunocompetence of the host is believed to be exceedingly important in the growth of transformed cells. In an immunocompetent host the initially transformed cells will not be allowed to grow and progress into a fatal tumor; while an immunosuppressed host may not be able to provide the degree of immune surveillance to prevent tumor development. We have undertaken studies with known chemical carcinogens given IT to define systems which may prove useful in the study of the effects of smoke inhalation at a later date.

a. Intratracheal Instillation of Carcinogen

Our findings indicate that one intratracheal instillation of 500μg MCA was not sufficient to produce immunosuppression in C3H/f Mai, C57BL/6 Cum and DBA/2 J mice of either sex when responses of spleen lymphocytes were tested in vitro three days after exposure (CTR-6A). The responses were unique to the group of mice tested, in that in one experiment they were suppressed and in another they were not. The kinetics (1, 2 or 3 weeks after exposure) of a single instillation was not determined. We did find that after several IT exposured of 500μg MCA that T-cell response was somewhat depressed and B-cell activity significantly so in C3H/f Mai mice (Demoise, Kouri and Whitmire, 1974).

These animals were, however, still capable of an enhanced spleen cell response when challenged with tumor-transplanted cells. It was also interesting that transplanted tumor cells grew slightly better in the MCA exposed mice and this was attributed to the lowered B-cell activity observed which indirectly suggested a lower level of circulating blocking antibodies.

Host tissue responses as animal, spleen, lung and thymus weights as well as total peripheral white blood cell counts also were not noticeably altered three days after only one intratracheal instillation of 500μg MCA. Again, the kinetics of this treatment was not determined. However, we did find after repeated weekly instillations that noticeable changes occurred such as a reduction in animal and thymus weights, an increase in lung weight and fluctuations in spleen weights and total numbers of white blood cells among the MCA exposed C3H/f Mai mice.

Comparative effects of repeated instillations of 500μg MCA and 1000μg diethylnitrosamine (DEN) on spleen lymphocyte and host tissue responses were studied in C3H/f Mai mice (CTR-6B). As previously observed, both T and B-cell responses of spleen lymphocytes were depressed by repeated instillations of MCA whereas after two and three instillations of DEN, the B and T-cell responses were enhanced. One instillation of 1000μg DEN, like a single 500μg instillation of MCA, was not sufficient to always produce immunosuppression when measured three days after exposure.

In preliminary studies of tumor transplant growth in DEN treated mice we have found no significant differences in growth rates but tumors transplant more readily in the DEN mice. Four and eight exposures to DEN and four exposured of MCA were sufficient to prolong the survival of allogeneic skin grafts. In general, the most tissue responses as animal, spleen, lung and thymus as well as total peripheral white blood cell counts from the DEN treated mice paralleled the changes observed in the MCA mice but were not as pronounced.

The intratracheal instillation of benzo(a)pyrene (BaP) and Fe_2O_3 dust in C3H/f Mai mice was observed to have altered the response of spleen lymphocytes when they were tested after two, six and eleven exposures (D050). Measurements of spleen cell activities, in general, showed (1) after two exposures, a depression of both T and B-cell responses, (2) after six exposures, a greatly enhanced T and B-cell activity, that was more pronounced in the B-cell population, (3) after eleven exposures, a distinctly depressed T-cell response and a much reduced B-cell activity compared to that observed after six exposures. These fluctuations in levels of competence were attributed to an initial depressive effect exerted by the chemical carcinogen, BaP, followed by an over-riding enhanced spleen cell activity exhibited by the host in response to BaP-transformed lung cells.

b. Subcutaneous Administration of Carcinogen

C3H/f Mai and C57BL/6 Cum mice have been exposed to 3-methylcholanthrene (MCA) in subcutaneous implants of wax pellets and the effect that this treatment has on the relationship between levels of host immunocompetence and tumorigenesis is being evaluated (CTR-46). Studies are now in progress that document the capacity of spleen lymphocytes to react to mitogenic stimulation, to neutralize tumor cells transplanted in vivo and to inhibit their growth in vitro at various times after MCA exposure.

B. Screening of Carcinogens

1. Subcutaneous Route

Earlier studies with MCA, DMBA and BaP have shown the C3H/f Mai strain to be the most susceptible to subcutaneous carcinogenesis. Based on these studies we undertook the screening of 14 fractions of 1R1 and 1A1 reference cigarette smoke condensates to determine their ability to induce tumors alone or with MCA as a cocarcinogen. In order to give as high doses of these fractions as possible, they were implanted as pellets using 1:1 beeswax:trioctanoin as a vehicle. This vehicle has advantages as well as disadvantages. Although it allows larger doses to be given without toxicity, the latency period for tumor development is extended. MCA (150μg) in trioctanoin induced 100% tumors in 20 weeks: while 150μg in beeswax:trioctanoin has produced only 43% tumors in 80 weeks. When 10μg MCA was given in trioctanoin, 38% tumors developed in 82 weeks; however, no tumors developed in the same period when 10μg was given in beeswax:trioctanoin. When 10μg MCA in trioctanoin was injected directly into the fresh beeswax:trioctanoin pellet, as was done with the CSC fraction, 15% tumors have developed in 82 weeks. Further studies to define the relationship between vehicle, carcinogen dose, and tumor latency are planned (CTR-19) using known carcinogens.

The 1R1 fractions have been on test for 82 weeks. Several tumors have developed with the fraction alone, but not at the site of injection. However, with MCA (10μg) as a cocarcinogen, as high as 75% tumors have been induced with the NCH fraction of 1R1. The 1A1 fractions have also been tested in a similar manner. No tumors have developed with the CSC fraction alone, however, with 10μg MCA we have obtained up to 50% tumors with B1a fractions. At this time, 1A1 appears to be less cocarcinogenic than the 1R1 fractions.

Fifteen whole cigarette smoke condensates were obtained through Dr. Gori at NCI. No tumors have occurred in mice receiving only the condensates. As cocarcinogens with 10μg MCA, tumor incidences range from 0-67%, while 15% tumors have occurred with the 10μg MCA control. The hand suckered CSC and the SEB1I were the most tumorigenetic by this assay system.

2. IT Route (CTR-9A, C, D)

1A1-CSC fractions and 15 whole CSC materials have been given IT to C3H/f mice in three injections and observed up to 64 weeks. Those animals which have been sacrificed have failed to demonstrate lung tumors on gross observation. These studies will be terminated over the next several weeks and histological examinations made. These early studies tend to indicate that lower, non-toxic doses should have been given over longer periods

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and possibly it may be necessary to add Fe_2O_3 or a cocarcinogen in order to produce tumors.

3. Wax Pellet Lung Implantation Assay (CTR-9B)

The use of the B:T implant procedure has two advantages over the IT route - i.e., it is a one time operation and larger doses are not toxic. This technique has been used to implant 8 mg whole CSC material in the lung of C3H/f mice. These mice are due to be taken off test during the next few weeks. If no tumors are found it may prove necessary to add a cocarcinogen as was done in CTR-1 for SC tumorigenesis with CSC material.

4. AHH Induction

IT instillation of CSC 1A1 fractions demonstrates that B_1a, B_1b, N_{MeOH} and N_{NM} are good inducers of pulmonary AHH. The whole 1A1 CSC, the reconstituted CSC and fraction N_{eH}, B_e and WA are weak inducers of pulmonary AHH.

The effects of the 1A1 fraction on BP metabolism in vitro was also tested and it was found that fraction B_1b, B_1a, N_{NM} and N_{MeOH} were potent competitive inhibitors of BP metabolism indicating these fractions contain chemicals which definitely influence pulmonary AHH activity.

The induction kinetics of pulmonary AHH following exposure of C57BL/6 mice to one 1A1 cigarette was shown to be rapidly induced and peak activity observed 6 hours post exposure. Liver AHH was unaffected. Exposure to seven consecutive cigarettes was very similar to that observed with one cigarette.

In vitro studies in conjunction with other contracts in our laboratory have studied the relative levels of AHH activities in various cell lines. Results indicate that certain cell lines can have measurable levels of AHH activity and their transforming potential may be related to this AHH activity. This finding may prove useful in the development of an in vitro carcinogenic screening procedure.

II SUMMARY PROPOSAL FOR CTR-14

July 1, 1975 - June 30, 1976

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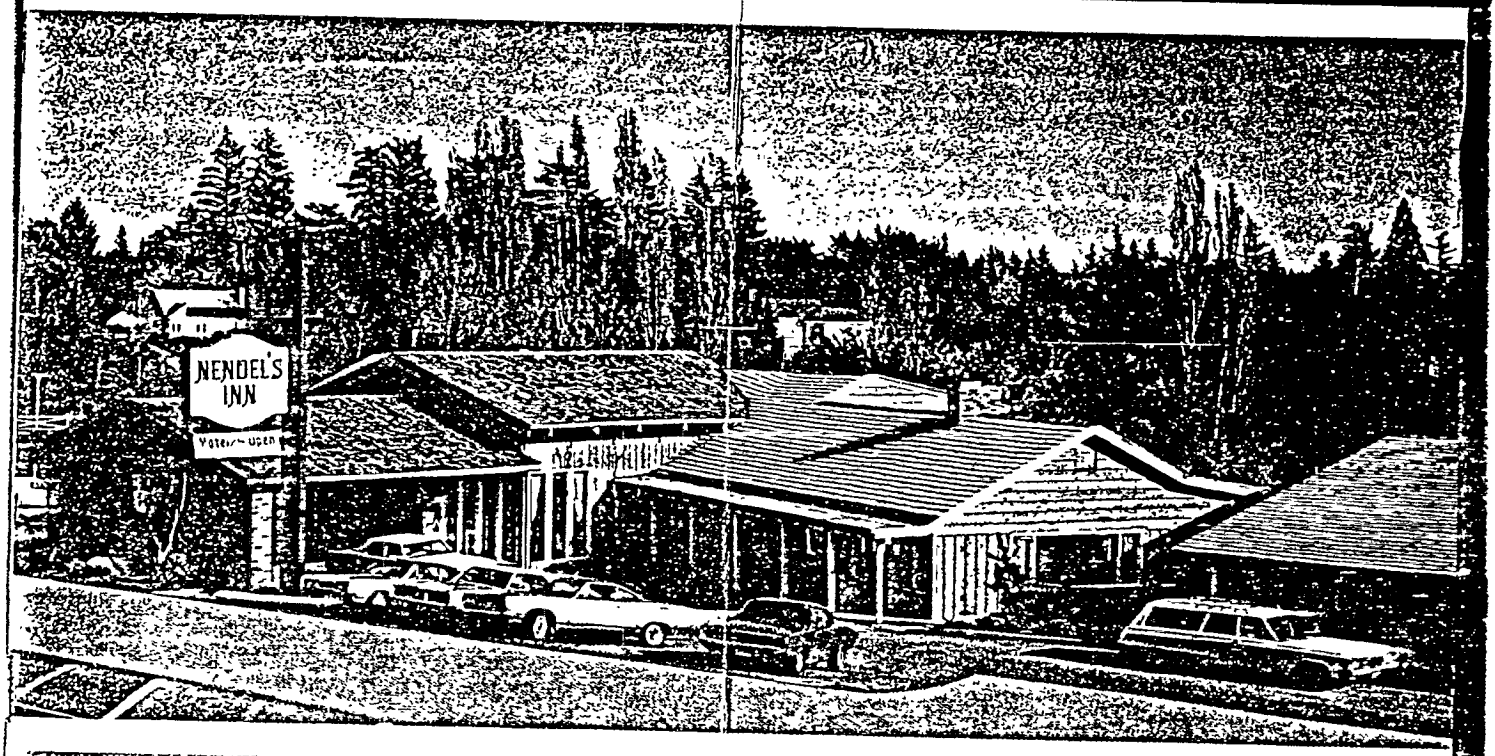

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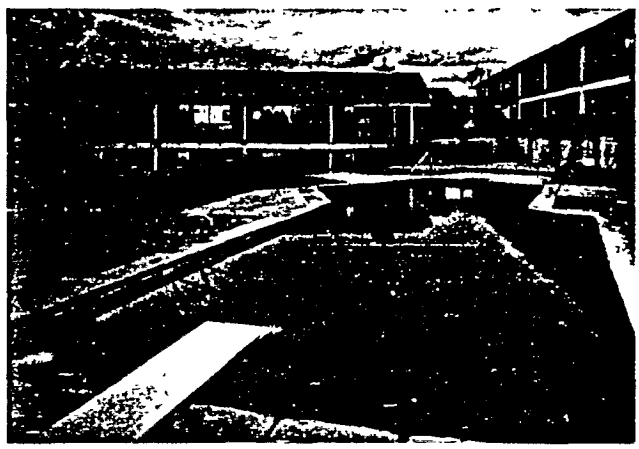

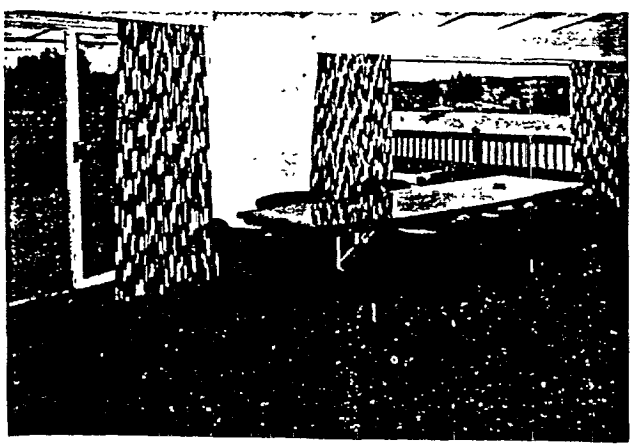

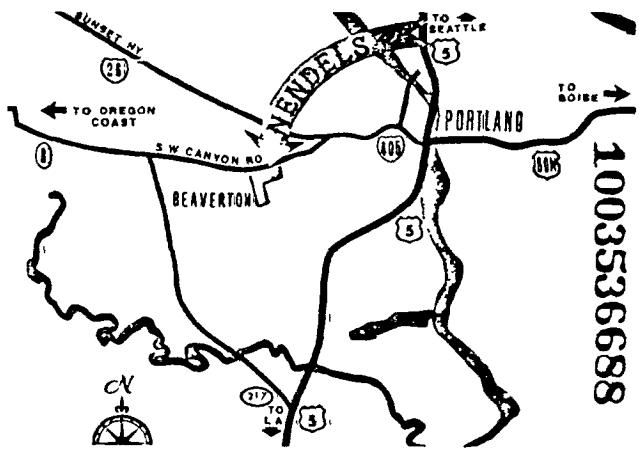

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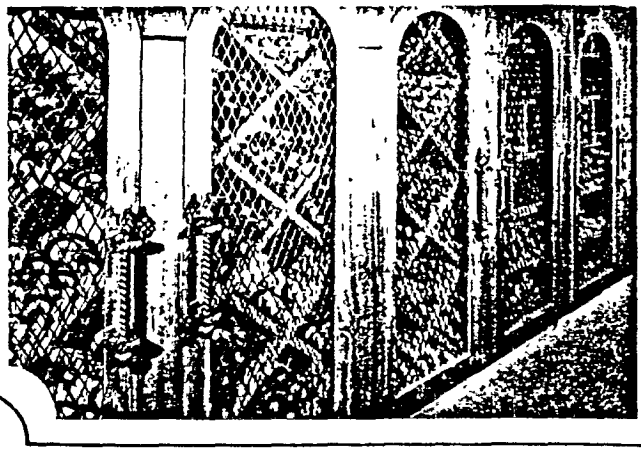

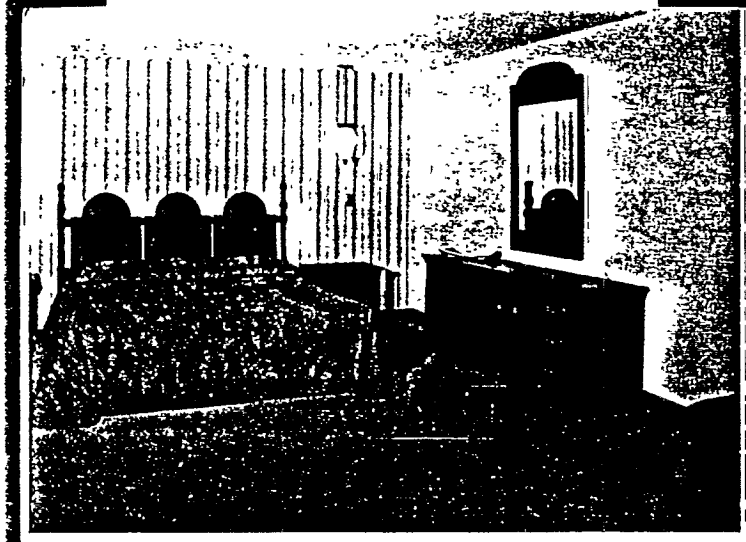

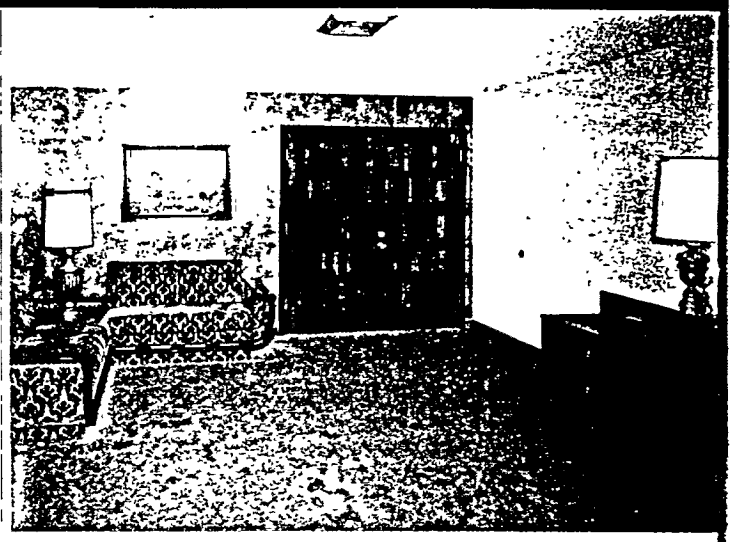

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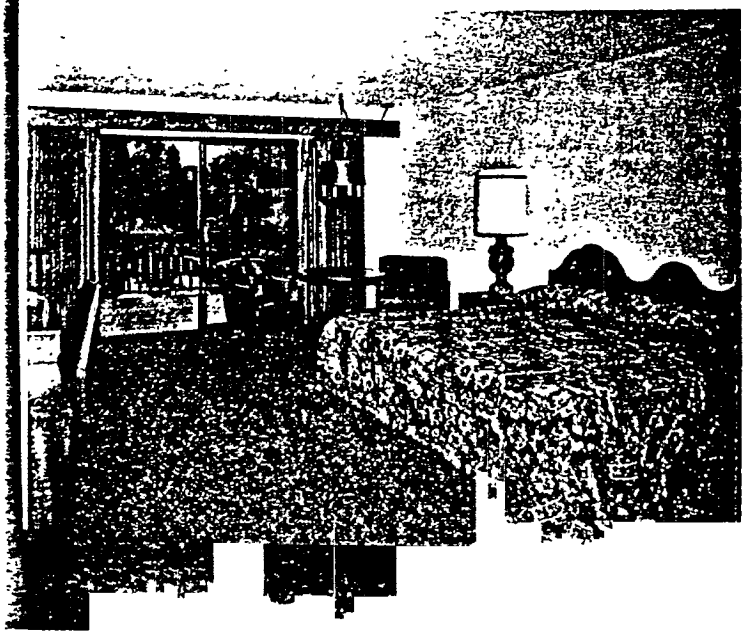

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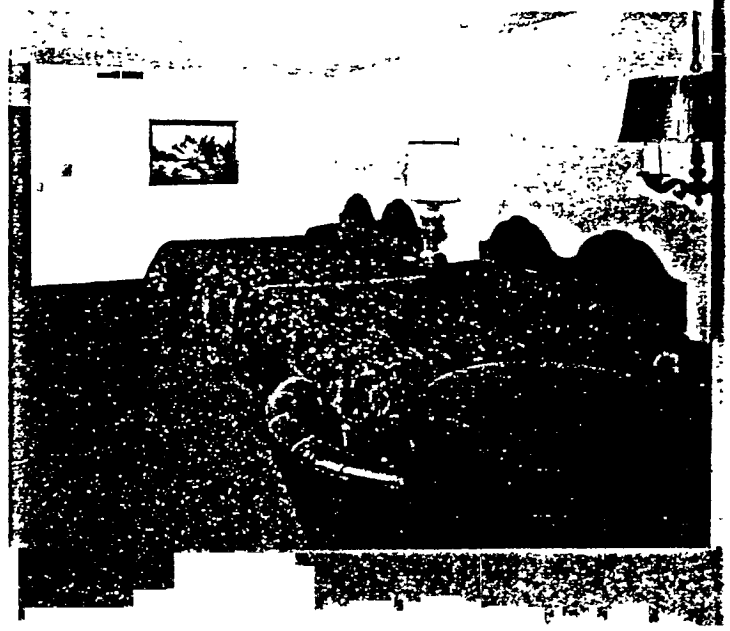


II. Summary Proposal for Contract Year July 1, 1975 - June 30, 1976 for CTR 14

The proposed studies for the new contract year have been divided into three sections for research and a support section dealing with computer handling of our data. No proposal of the histological work is presented as this represents a classic type service to all the research and bioassay on the CTR programs at MA.

Section A: Chemical Carcinogenesis Assay Systems - C.E.Whitmire, Ph.D.

This contract year will be used to complete the ongoing studies evaluating the MCA & BaP-IT lung carcinogenesis studies. This will demonstrate the best dose and schedule for administration of a known carcinogen and give us leads to be applied in using this system in assay of the carcinogenic potential of CSC materials. We will undertake new studies to determine the early lesions and onset of tumors by serial killing of mice treated with MCA and BaP. These will be useful in evaluating the immunological findings related to the effects of chemical carcinogens found by Dr. Demoise & Dr. Levy. These studies will also establish both the dose and the time of administration of BaP or MCA as a cocarcinogen by the IT route for study with CSC materials or whole smoke.

The use of phorboll esters as a promoter in MCA lung carcinogenesis will be undertaken and if shown useful will be adapted to the assay of CSC materials. The use of Fe_2O_3 in the lung assay of CSC is also a possible approach to be undertaken.

Other studies will evaluate the use of the wax pellet lung implant in the assay of CSC materials. We will also continue our studies of the SC pellet procedure as an assay of CSC materials. These studies have shown promise during the past year and need to be evaluated as to their reproducibility.

Section B: AHH Related Chemical Carcinogenesis, - R.E.Kouri Ph.D.

The completion of the studies proposed for this contract year should go a long way in determining those conditions effecting and/or altering the susceptiblity of pulmonary tissue to chemically-induced cancers. This part of the contract will concentrate on one particular parameter that is known to play a major role in this neoplastic response, the enzyme system aryl hydrocarbon hydroxylase (AHH). The studies will center on the effects of inducers and/or inhibitors of pulmonary AHH (and

epoxide hydrase) simultaneously with their effects on 3-methylcholanthrene-induced lung carcinomas. The inducing chemical will be the herbicide by-product, TCDD, and the inhibiting chemicals will be 7,8-benzoflavone and trichloropropane oxide. The flavone inhibits "induced" or P-448-mediated AHH, while the oxide inhibits the enzyme system epoxide hydrase. The effects of particular fractions of the IAI condensate will be similarly studied. Certain ongoing studies will probably continue into the new contract year. The studies concerning the effects of whole cigarette smoke on pulmonary AHH will continue in order to observe such parameters as 1) effect of type of smoking machine, 2) effect of diet, 3) effect of type of cigarette, 4) effect of dose of exposure, and 5) effect of AHH genotype. Studies on the role of genetically regulated levels of pulmonary AHH in chemically-induced lung carcinomas will also continue.

Section C: Chemical Carcinogenesis of the Lung - C.F. Demoise, Ph.D.

The studies of the new contract year will include a collaborative study in which the lung tumor induction of MCA is undertaken in Section A to compare with the serial sacrifice of mice for histological finding by Dr. Sass. Animals from the same study will be studied by Dr. Levy at Scripps for immunocompetency and by this section for cellular immunocompetency by various techniques developed during the past year. This collaborative study will lead to better understanding of the relationship of initiation and development of lung tumors by carcinogens entering the lung. These studies will provide information useful in designing future smoke inhalation studies using MCA or BaP as cocarcinogens. The immunological procedures will be applied to future smoke inhalation studies also.

In addition to these studies the effects of immunosuppressive drugs will have on the latency period of MCA induced tumors of the lungs. Studies during the past year with weekly vs. biweekly MCA treatments have demonstrated the biweekly schedulle to give tumors in a shorter period of time. Based on the studies at Scripps the possibility of this shorter latency period is believed to be due, at least in part, to the longer period of immunosuppression induced by MCA.

A new approach will be undertaken to develop a model system for nitrosamine lung carcinogenesis and new studies will be undertaken to develop an intratracheal cocarcinogenesis or two stage carcinogenesis bioassay for CSC materials.

Section F: Computer Data Handling - Miles J. Haven, M.S.

During the new contract year this section will be involved in increasing the capacity to handle more experiments as well as more observations per animal during the period on test. New summary reports and analysis of data will be undertaken. This will include the generation of graphic plots for better data analysis.

III PROPOSED STUDIES

SECTION A

CHEMICAL CARCINOGENESIS

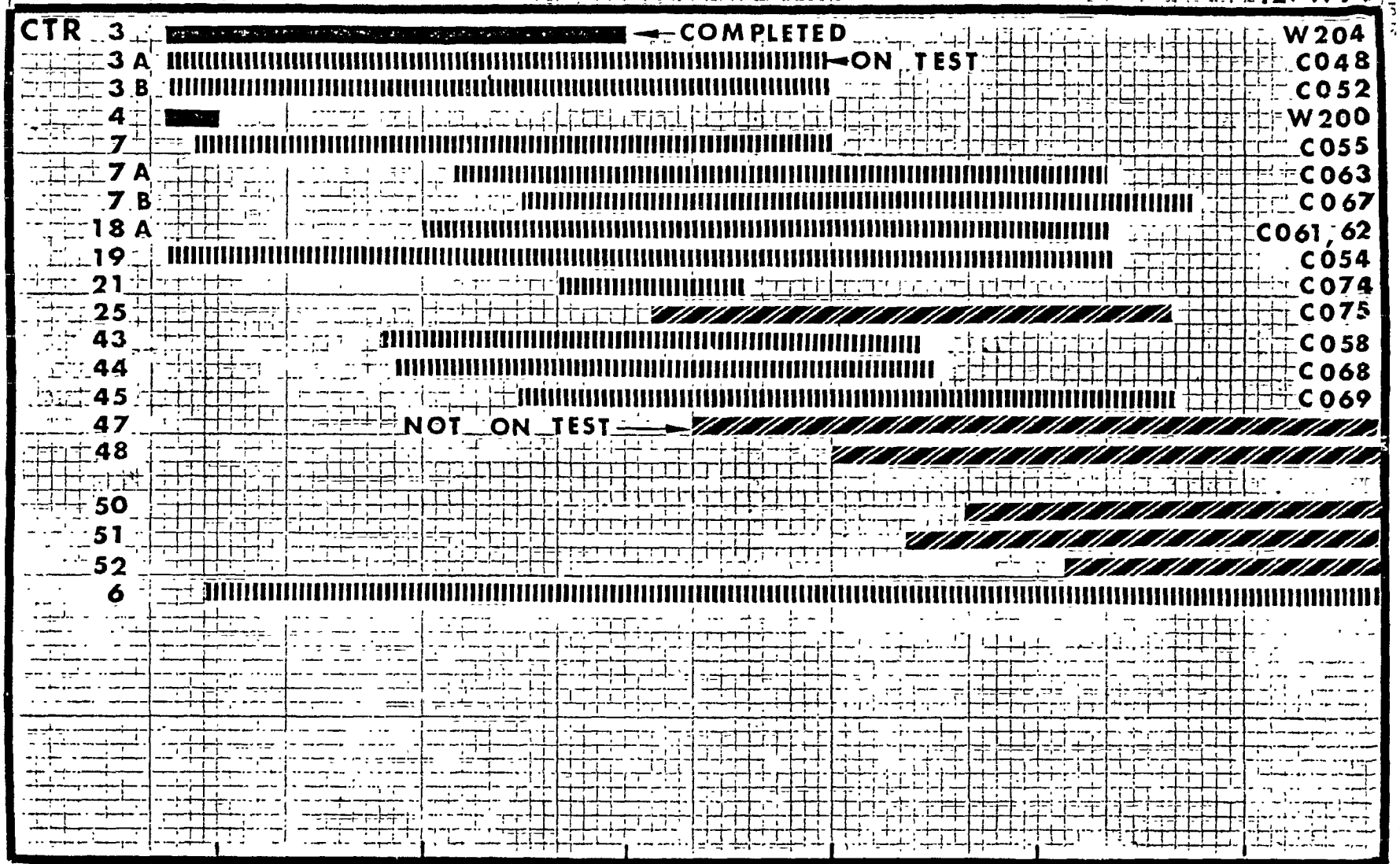
C.E. Whitmire, Ph.D.

SECTION A SCHEDULE December 1, 1974

CTR #	MA #	EXPERIMENT
CTR-3	(W204)	IT-MCA in C57BL, C57BL/6, C3H/Anf, BC3F1.
CTR-3A	(C048)	IT-MCA in C3H/f Mai, gel vs. trioctanoin, weekly vs biweekly injections.
CTR-3B	(C052)	IT-MCA in C57BL/6, gel vs trioctanoin, weekly vs biweekly injections.
CTR-4	(W200)	SC-MCA comparison of C57BL, C57BL/6, C3H/Anf and BC3F1/Cum mice.
CTR-7	(C055)	IT-MCA in C3H/f Mai, 6x weekly vs 12x weekly, 62.5, 125, 250μg.
CTR-7A	(C063)	IT-MCA in C3H/f Mai, 6x vs 12x weekly, 62.5, 125, 250μg.
CTR-7B	(C067)	IT-MCA in C3H/f Anf, 1x, 2x, 4x, 6x/week, 9.38, 18.75, 37.5, 75.0, 150, 300μg doses.
CTR-18A	(C061 & C062)	DMN in B:T pellets, in C3H, C57BL/6, BALB/c, DBA/2, SWR/J mice (AHH + vs -).
CTR-19	(C072)	SC-B:T vs trioctanoin of MCA, BP and DMBA in C3H/f, C57BL/6, BC3F1 and DBA/2 mice.
CTR-21	(C074)	Phorbol ester IT toxicity and AHH inducibility.
CTR-25	(C075)	IT-Phorbol + MCA lung carcinogenesis.
CTR-43	(C058)	IT-BaP + Fe_2O_3, C3H/f mice.
CTR-44	(C068)	IT-BaP + Fe_2O_3, DBA/2 mice.
CTR-45	(C069)	DEN, DBN, PYR, PIP - B:T wax lung implants in C3H/f mice.
CTR-47	1975 - 1976 period	CSC-SC-cocarcinogenesis assay development.
CTR-48		CSC-pellet-cocarcinogenesis assay development.
CTR-50		BaP serial sacrifices for early carcinogenic changes detection.
CTR-51		MCA serial sacrifices for early carcinogenic changes detection.
CTR-52		CSC-IT-Phorbol ester promotor assay development.
CTR-6		Scripps collaborative studies.

SECTION A SCHEDULE

12/1/74



A. Proposed Studies for the Contract Year

1. Completion of ongoing experiments

a. Intratracheal lung carcinogenesis model

CTR-7, 7A, 7B, These studies are designed to establish the best dose and schedule for inoculation of MCA in the development of the IT lung carcinogenesis model.

CTR-25, This study was designed to develop an animal model system which closely parallels the skin painting assay of carcinogens in which an initiator and promoter are used. It is hoped that phorbol esters will provide a rapid means of producing squamous cell carcinomas in the lungs of mice with low doses of good carcinogens or with higher, non-toxic doses of weak carcinogens.

CTR-43, 44, These studies are concerned with the use of BaP with and without Fe_2O_3 for lung tumor development. They will be completed and new studies probably initiated to further define this assay system.

b. Wax-pellet lung implant carcinogenesis model

CTR-18A and 45, These have been undertaken to develop a system whereby the nitrosamine can be studied in lung carcinogenesis. The pellet implant procedure was undertaken for two reasons: first, it offers a safe way for handling DMN and secondly it allows larger doses of weak carcinogens to be used.

c. Wax-pellet subcutaneous carcinogenesis with known carcinogens

CTR-19, This will be completed to give us a better understanding of this model system which has been shown to be useful for CSC materials in CTR-1.

2. Proposed new studies for the contract year.

During the new contract period this section will be concerned primarily with the development of model assay systems for the assay of tobacco related carcinogens. Efforts will be made to refine the SC cocarcinogenesis assay and to apply this knowledge to the B:T wax pellet lung assay procedure. We will also apply our IT route of inoculation to the assay of CSC materials.

In addition to these assay procedures, we will continue to define the IT procedures for MCA and BaP-by serial sacrifice studies to determine how early the tumors occur and where these tumors arrive.

New collaborative studies with Scripps Institute have been designed to determine the relationship of a single MCA dose ranging from 10μg to 600μg to immunocompetence in the C3H/f mice. These studies will be followed by multiple IT instillations of MCA in which the immunocompetence will be followed and compared to the histopathological findings during the treatment period and during the period when tumors are developing.

CTR-6 December 1, 1974

Collaborative Study with Drs. Levy & Lerner at Scripps Clinic and Research Foundation.

Objectives:

To study the immunosuppressive effects of MCA given intratracheally to C3H/f mice.

Procedure:

Mice are given MCA by IT injection at Microbiological Associates, and shipped to Scripps where they are challenged with goat RBC to determine their immunocompetence.

Progress:

#1. During the past year C57BL/6 Cum, C3H/f Mai and DBA/2J mice have been tested for their response to 500μg MCA. These studies showed that immunosuppression occurs in the C57BL/6 and C3H/f but not the DBA/2 mice. These results would indicate the ability to metabolize the MCA may play a role in the immunosuppressive potential since DBA/2 mice are AHH non-inducible.

Proposed Studies:

#1. Studies will be initiated in January 1975 to determine the rellative immunosuppressive effects of various doses of MCA in the C3H/f mice.

#2. A study will be initiated later in the year to follow the immunosuppressive effects of multiple MCA injections. The mice will be followed after the injections have been stopped to determine the effect of tumor development. This study will parallel CTR-51 which will define the histological findings of early lesion development.

CSC and CSC fraction subcutaneous cocarcinogenesis - assay development.

Objectives:

In previous studies, we have shown a difference in the carcinogenic potential of various whole CSC and fractions can be detected by SC cocarcinogenesis with MCA. There have, however, been differences in the tumor incidences of two IRI CSC tested. This could be due to the differences in these two CSC or in the way they were handled. It is proposed that several whole CSC and several CSC fractions be tested again by this procedure in three consecutive tests to determine the reproducibility of this assay procedure.

Procedure:

Samples would be assayed by the SC route using 10μg MCA and/or a low dose of BaP as a cocarcinogen. The cocarcinogen will be given as previously as a second injection into the pellet area as well as by combining directly with the pelleted CSC material. As a further check on the reproducibility of the assay system, each sample should be assayed at two dilutions to determine the relationship of dose to tumor induction. The B:T ratio used should be 1 part beeswax to 1 and 2 parts trioctanoin.

The C3H/Anf Cum mouse should be used for these studies and they should be terminated at 15 months, since little was gained by holding the previous studies for 18 months.

Cocarcinogenesis using pellet implantation of CSC and CSC fractions in the lung as a carcinogenesis assay procedure.

Objectives:

SC studies with CSC-MCA pellets have demonstrated the necessity of a cocarcinogen to obtain a tumor at the site of injection. This may account for the lack of tumor induction by lung B:T-CSC pellets during the past year. It is suggested that a limited number of CSC and CSC fractions be given with 10μg MCA as pellets to determine if this assay system could be adapted to lung tumor studies with weak and multiple carcinogens present in CSC.

Procedure:

C3H/Anf Cum or B6C3F1 mice will be injected with high, but nontoxic, doses of CSC combined with 10μg MCA or a low dose of BaP in pellets. The mice will be sacrificed at 15 months or when death is imminent and gross and histological examinations made for lung and other tumors. The CSC materials used in CTR-1 provide evidence as to which samples would prove useful for the development of this assay system.

Serial sacrifice for early carcinogenic change due to BaP given IT in C3H/f mice.

Objectives:

In the experiments now on test, we have found numerous BA lesions, and squamous metaplasia which are difficult to classify as squamous cell carcinomas in the absence of metastasis. The only gross indication that lung tumor may have developed in an animal is its general appearance, which may be deceiving. Some animals will become apparently ill with small lung lesions, while others will have massive lung tumors indicating they have had tumors for a considerable period prior to detection. The detection of malignant tumors earlier will of course shorten our experiment time, however, the actual development of tumors is of significance in our immunological studies.

Procedure:

C3H/Anf Cum or B6C3/F1 Cum mice will be given 1.2mg BaP and 0.6mg Fe_2O_3 in 6 biweekly intratracheal injections. Ten mice will be sacrificed at two week intervals starting at the time of the second IT injection. The lungs will be examined grossly for the presence of tumors and fluorescence with a black light. A portion of the lung of 3-5 mice will be minced separately and transplanted subcutaneously to determine at what point in time malignant transformation has occured. This will be correlated with the histological finding of all 10 mice. The spleen and thymuses will be weighed to determine gross changes occurring in the RES system. The spleens of some animals will be used for possible cellular immunological studies based on transplant and histological studies. When frank tumors are grossly detectable in 70% of the mice serially sacrificed for two consecutive periods, the experiment will be terminated and all remaining mice will be sacrificed.

If transplants are successful in the early period during continued treatment with BaP, it may be related to a carry-over of BaP and not necessarily due to the presence of transformed cells. This possibility exists for exceedingly long periods if the yellow appearance of the lungs in our present studies can be considered to be residue BaP. It must also be remembered that the Fe_2O_3 will also be present in the transplanted tissues and may act as a local irritant. The histological findings of the lung tissue will be correlated with the ability to transplant the lung tissue. If the residue BaP does not prove to be a carcinogenic factor, then we may be able to determine at what stage the squamous metaplasia can be considered to be a squamous CA in the absence of metastasis.

CTR-51 December 1, 1974

Repeat of CTR-50 using MCA to induce lung tumors by IT inoculation.

Objectives:

This study will answer some of the same questions as the BaP experiment. It is felt essential it be done with MCA also since we are using the MCA system for determining the immunological suppressive effect in the Scripp's studies. We will be shipping mice to them at varying intervals after inoculation for immunological studies and these should be correlated with histological findings to make their findings meaningful. Studies will be undertaken in our laboratory to correlate with cellular immunity.

Procedure:

Same as CTR-50 only MCA will be substituted for BaP and no Fe_2O_3 will be used.

Phorbol ester promoter effects with CSC and CSC fractions given by IT route for lung carcinogenesis assay development.

Objectives:

To apply the well known use of the promoter action of phorbol esters to lung carcinogenesis it is suggested the nontoxic dose established in CTR-21 be combined with CSC and CSC fractions given IT to produce lung tumors. This study will only be undertaken in the event PE proves usefull with MCA (CTR-25).

Procedure:

BC3F1 or B6C3F1 mice will be treated with low doses of CSC at biweekly intervals with phorbol ester being administered on a biweekly schedule. Such a schedule would give phorbol ester in weeks 1, 3, 5, etc. and the CSC in weeks 2, 4, 6 etc. In this way, lung tumors may be produced in much the same way as skin tumors are produced in the standard in vivo bioassay now in use.

SECTION B

AHH RELATED CHEMICAL CARCINOGENESIS

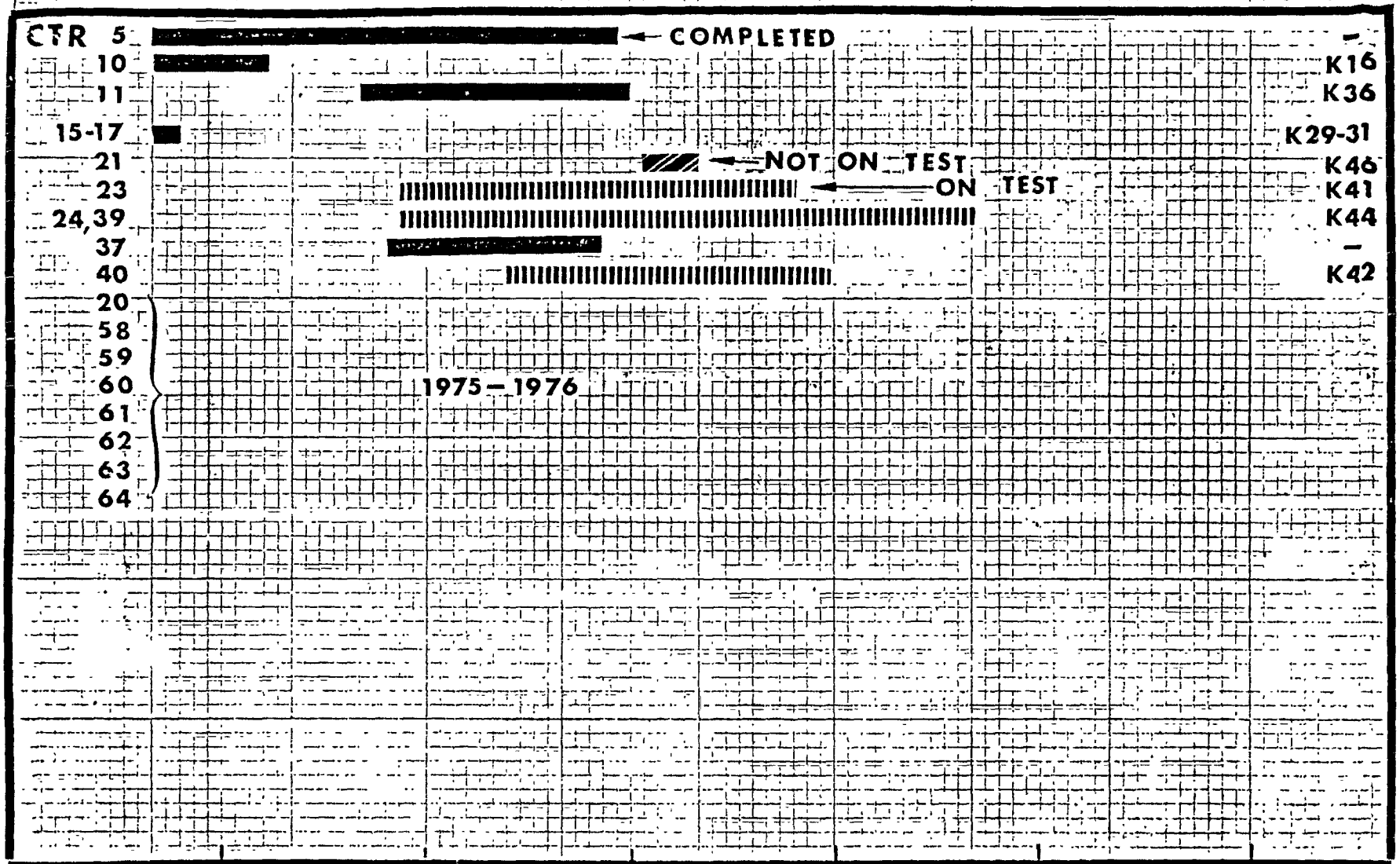
R.E. Kouri, Ph.D.

SECTION B SCHEDULE December 1, 1974

CTR #	MA #	Experiment
CTR-5		IT-MCA in C57BL/6, DBA/2 and B6D2F1
CTR-10	K-16	Effects of 1A1 CSC fractions on AHH activity.
CTR-11	K-17,39	Kinetics of pulmonary AHH activity with 1A1 smoke exposure.
CTR-15,17	K-29,31	TCDD effects of MCA-SC carcinogenesis.
CTR-21	K-47	Phorbol ester pulmonary AHH induction (mouse studies in Section A, AHH assay in Section B)
CTR-23	K-41	SC-MCA in C3H/f x DBA/2
CTR-24,39	K-44	IT-MCA in C3H/f x DBA/2
CTR-36-37		In vitro chemical carcinogenesis with C3H 3T 10 1/2 cells.
CTR-40	K-42	SC-MCA TCDD studies in DBA/2 mice (repeat of CTR 15,17).
CTR-20	K-46	DMN effect on pulmonary AHH induction.
CTR-58		Effects of TCDD on pulmonary AHH activity
CTR-59		Effects of TCDD on lung cancer induced by MCA
CTR-60		Effects of 7,8-benzoflavone on pulmonary AHH activity induced by MCA
CTR-61		Effect of 7,8-benzoflavone on pulmonary tumors induced by MCA
CTR-62		Effect of triochloro-propane oxide (TCPO) on pulmonary AHH and epoxide hydrase (EH) activity
CTR-63		Effects of TCPO on lung tumorigenesis induced by MCA
CTR-64		To observe the role of particular fractions of the 1A1 condensate on lung tumors induced by MCA

SECTION B SCHEDULE

12/1/74



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A. Proposed Studies for the Contract Year.

1. Completion of ongoing experiments.

a. Effects of various substances on lung AHH induction.

CTR-11 Kinetics of pulmonary AHH activity with 1A1 smoke exposure.

CTR-20 DMN effect on pulmonary AHH induction.

CTR-21 Phorbol ester pulmonary AHH induction (Mouse studies in Section A, AHH assay in Section B).

b. Intratracheal lung carcinogenesis.

CTR-24 IT-MCA in C3H/f x DBA/2

c. Subcutaneous Carcinogenesis

CTR-23 SC-MCA in C3H/f x DBA/2

d. Effects of AHH inducers on chemical carcinogenesis.

CTR-40 SC-MCA TCDD studies in DBA/2 mice (Repeat of CTR 15, 17)

CTR-20

Effect of Dimethylnitrosamine (DMN) on Pulmonary AHH Levels

Objectives:

Observe the effects of DMN or MCA pretreatment on ability of inducible and noninducible mice to metabolize these carcinogens.

Background:

DMN is a potent chemical carcinogen which is found in small quantities in cigarette smoke and may be formed in situ by nonenzymatic reactions involving inorganic nitrite and amines. Results suggest that DMN must be metabolically activated to its carcinogenic form and the enzyme causing this activation is DMN-demethylase. The presence of this enzyme bears a mirror-image relationship to the presence of the AHH enzymes. Compounds which induce high levels of AHH seem to be potent repressors of DMN-demethylase activity. Thus, animals which are noninducible for AHH activity should be more sensitive to DMN-induced tumors. This conclusion is based on the fact that (1) DMN may be an inducer of AHH activity, (2) DMN would repress DMN-demethylase activity, in inducible animals, (3) DMN would have no effect on DMN-demethylase activity in noninducible animals. The in vivo study designed to test this hypothesis is in CTR-31. Certain biochemical tests should predate this test and these are given in this protocol. Some of these studies may involve collaboration with Dr. J. Arcos, Tulane University, New Orleans, Louisiana.

Procedure:

a. Groups *(3 mice per group)

Treatment	Strains B6	Strains D2
DMN	*3	3
MCA	3	3
DMN + MCA (given simultaneously)	3	3
DMN + MCA (DMN 24 hrs. previous to MCA)	3	3
DMN + MCA (MCA 24 hrs. previous to DMN)	3	3
Untreated	3	3

b. Protocol
(1) Determine if DMN-demethylase activity is measurable in the lungs of B6 and D2 mice. If so, then all assays will follow intratracheal administration of the chemicals. If not, then all assays will follow intraperitoneal administration and will be performed on hepatic rather than pulmonary tissue.

(2) Determine dose effects of DMN on demethylase and AHH activity. Use dose of DMN giving maximum induction of AHH in the B6 animals.

Progress:

The initiation of this study is awaiting some recent data from the laboratories of Drs. J. Arcos and M. Arcos. These experimentors are trying to assay for DMN-demethylase activity in lung tissue and the techniques are presently being improved.

CTR-21

Effects of Promotors on Pulmonary AHH Activity

Objectives:

To establish whether phorbol ester alters levels of pulmonary AHH activity.

Background:

Promotors are substances with the property of increasing the carcinogenic effect of other compounds. Recent data suggest that the mechanism of action of promotors may be related to the fact that all of these compounds are very good inhibitors of DNA repair. Compounds such as phorbol esters, vitamin A, or particular steroid hormones are examples of chemicals which inhibit DNA repair and are potent promotors of chemically-induced cancers. Since both vitamin A and steroid hormones directly influence AHH activity, there may be a relationship between promoting activity, repair inhibition, and AHH enzyme levels.

Procedures:

a. Groups

Treatment	Time (Days) 1	7	14	21
PE	3	3	3	3
PE + MCA	3	3	3	3
MCA	3	3	3	3
Control	3			

*Number of animals.

b. Protocol
(1) Determine a dose of PE that when given IT (in 0.02 ml) will kill about 10% of the animals in 21 days (LD_{10-21}). The dose will probably be about 0.01μg per mouse.
(2) At this dose level, give PE twice a week and observe constitutive and MCA induced AHH levels at the times indicated.
(3) Repeat for both strains of mice.

Progress:

Determination of the LD(10-21) is presently being determined; the available results are shown in CTR-21. The AHH studies should begin by February-March, 1975.

2. New experiments.

Introduction: Recent results using our SC mouse model system for carcinogenesis of MCA has demonstrated that not only is susceptibility to chemical carcinogenesis in inbred strains of mice regulated to a major degree by the genetic regulated levels of AHH, but also, this genetic regulation can be altered somewhat by the use of other chemicals which can alter AHH regardless of genotype (e.g. TCDD) Of particular importance, is the fact that the treatment schedules for TCDD and MCA play a major role in this altered sensitivity. Co-treatment of MCA and TCDD enhances the tumor response in MCA-non-responsive mice (e.g. DBA/2), however a 48 hour pretreatment with TCDD does not effect MCA tumorigenesis. This is somewhat surprising in that a 48 hour pretreatment will mean that there is maximal AHH activity at the time of MCA administration and one may think that this schedule should yield a maximum tumorigenic response. The fact that this was not the case suggests that AHH activity by itself is not the determining factor, but rather when the activity is increased is important.

Our rather simplistic working hypothesis explaining these results is that there are definite differences between the kinetics of induction of the epoxide-forming enzymes (e.g. AHH) and the epoxide-degrading enzymes (e.g. epoxide hydrase (EH)). During the first 24 hours, the levels of the two enzymes are comparable. Thus, we feel that during the 1st 24 hours, the epoxide has a longer effective lifetime and it is during this time that the neoplastic process is initiated. This hypothesis is very difficult to prove, or disprove, using the SC tumorigenesis system because AHH activity cannot be effectively quantitated in the tissue in which the tumor originates (e.g. connective tissue).

However, with the advent of the carcinogenesis system utilizing the IT administration of MCA to induce pulmonary carcinomas, this hypothesis can be tested. The lung provides the unique situation of being able to quantitate the enzymatic responses in the same tissue in which the tumor eventually occurs. Thus, observing the effects of inducers and/or inhibitors of pulmonary AHH simultaneously with their effects on MCA-induced carcinomas should allow for the determinations of these conditions that maximize susceptibility to lung cancer. These are the conditions that must be utilized to ascertain the relative carcinogenic as well as co-carcinogenic effects of whole cigarette smoke. Cigarette smoke may function as a co-carcinogen by inducing AHH in a manner analogous to TCDD. Thus, it may not be the actual amount of AHH

activity that is induced by cigarette smoke, but the relative time between induction and exposure to other known chemical carcinogen pollutants that is important to cancer production. Likewise, cigarette smoke may be a complete carcinogen, but, because of its fairly weak nature, almost perfect conditions must be present before its carcinogenic activity can be observed. Pre-, post- or co-treatment with an inducer of AHH may give these conditions. The experiments outlined in the following section should go a long way in determining these conditions.

Effects of inducers of AHH.

CTR-58 Effects of TCDD on pulmonary AHH activity.

Objective: Preliminary results from CTR-40 suggest that the inherent susceptibility to MCA carcinogenesis may be altered via the use of a chemical which induces AHH in strains of mice nonresponsive to MCA alone. Thus, the potential susceptibility of an individual may be determined by not only its genetic mechanism regulating AHH, but also the exposure to other chemicals whose effect may mitigate this genetic regulation. These studies (CTR-58 & 59) are designed to observe the effects of TCDD on the susceptibility of AHH-responsive and non-responsive mice to MCA-induced lung carcinomas.

Materials:

(1) Mice

(a) C3H/Anf Cum, ♀, 8-10 weeks old
(b) C57BL/6 Cum, ♀, 8-10 weeks old
(c) DBA/2 Cum, ♀, 8-10 weeks old

(2) TCDD in dioxane at concentrations of 100nM, 10nM, 1nM, 0.1nM.

Method:

(1) IT instill 0.02ml to 5 mice of each strain

(2) At 24 hours, kill by cervical dislocation, remove livers and lungs and assay for AHH activity.

(3) For kinetics, determine minimal dose of TCDD capable of inducing pulmonary, but not hepatic, AHH and observe amount of enzyme at 2, 4, 6, 12, 24, 48, 72 and 96 hours post treatment.

(4) Epoxide hydrase activity may also be monitored in these same tissues.

10035362 15

CTR-59 Effects of TCDD on lung cancer induced by MCA.

Objective: To determine the role of TCDD-effected levels of pulmonary AHH in MCA induced pulmonary carcinomas. These studies are based on the findings in CTR-40 and CTR-58.

Materials:

(1) Mice

(a) C57Bl/6 Cum (or C3H/Anf Cum), ♀, 8-10 weeks old

(b) DBA/2 Cum, ♀, 8-10 weeks old

(2) TCDD (as dose level determined in CTR-58)

(3) MCA at 250μg/0.02ml 0.2% gelatin in sterile saline.

(4) TCDD and MCA in same vehicle.

Groups:

(1) For C57Bl/6 mice (or C3H)

Treatment:

	-2 days	0 days	# mice
1.	Diox	Trioc	50
2.	TCDD	Trioc	50
3.	None	MCA	100
4.	Diox	MCA	100
5.	TCDD + MCA	None	100
6.	TCDD	MCA	100
7.	TCDD + MCA (same vehicle)	None	100

(2) Repeat groups for DBA/2 mice.

Procedures:

(1) Follow protocol, and give MCA 1, 2 or 3 times, (depending on results of ongoing experiments).

(2) At 2 months post treatment take 5 animals per group off test and observe macro and microscopically for lung tumors.

(3) When tumors found, take off remaining animals and observe for pulmonary tumors.

Effects of inhibitors of AHH activity.

CTR-60 Effects of 7,8-benzoflavone on pulmonary AHH activity induced by MCA.

Objective: 7,8-BF is a potent inhibitor of "induced" (or P-448)-mediated AHH activity. What would be the effects of this compound on pulmonary AHH? Under what conditions are maximal inhibitions observed? The use of this compound should decrease the amount of intracellular epoxide, the hypothetical ultimate carcinogen, but competitively inhibiting the metabolism of MCA.

Materials:

(1) Mice: C3H/Anf Cum, ♀, 8-10 weeks old

(2) 7,8-BF dissolved in 0.2% gelatin in sterile saline, or dissolved in trioctanoin.

(3) MCA (200μg/0.02ml gelatin solution)

Procedures:

(1) IT instill 200μg MCA plus various amounts of 7,8-BF in the same vehicle (total volume 0.02ml).

(2) Doses of 7,8-BF should be 0, 50, 100, 200 and 500μg.

(3) At 24 hours after treatment remove livers and lungs and assay for AHH activity.

(4) At dose level, giving maximum inhibition determine the kinetics of this inhibition.

CTR-61 Effect of 7,8-BF on pulmonary tumors induced by MCA.

Objective: To observe the biological consequences of the results observed in CTR-60. The conditions that allow for a decreased amount of intracellular epoxide should definitely influence the relative carcinogenicity of that particular dose of MCA.

Materials:

(1) Mice: C3H/Anf Cum, ♀, 8-10 weeks old

(2) 7,8-BF at concentration determined by CTR-60.

(3) MCA at 200μg/0.02ml gelatin solution.

Groups:

Treatment	Animals
Trioc or Gel	50
BF	100
MCA	100
BF + MCA	100

Procedures:

(1) Follow treatment schedule determined in CTR-60 to yield maximum pulmonary AHH inhibition.

(2) At 2 months post-treatment, take 5 animals off test and observe macro- and microscopically for lung tumors.

(3) Repeat every month until tumors observed.

(4) Record incidence as well as latency of tumor formation.

CTR-62 Effect of trichloro-propane oxide (TCPO) on pulmonary AHH and epoxide hydrase (EH) activity.

Objective: TCPO is a potent inhibitor of EH activity. This enzyme degrades epoxides to a noncarcinogenic end product, the dihydrodiols, and thus can affect the instantaneous level of this carcinogenic intermediate. Can this chemical affect AHH activity and EH activity *in vivo*?

Materials:

(1) Mice: C3H/Amf Cum, ♀, 8-10 weeks old

(2) MCA at 200μg/0.02ml gelatin solution.

(3) TCPO at various concentrations and suspended in the MCA vehicle.

Procedures:

(1) Determine relative toxicity of TCPO when given IT. (It is a very toxic compound.)

(2) Determine level of TCPO that when given with 200μg MCA, will alter AHH activity and inhibit EH activity at 24 hours post treatment.

(3) Determine the kinetics of this inhibition.

(4) May have to look at the spectrum of metabolites using thin layer chromatography, (in collaboration with Drs. Wang and Rasmussen) in order to be certain that the actual level of epoxide has increased, and not that the level of another metabolite has increased.

CTR-63 Effects of TCPO on lung tumorigenesis induced by MCA.

Objective: To observe the tumorigenic consequences of CTR-61. The use of TCPO should increase the level of epoxide by decreasing the rate of degradation to the non-carcinogenic dihydrodiols. This should enhance the tumorigenic effects of that particular dose of MCA.

Materials and Procedures are similar to that described in CTR-60.

Effect of fractions derived from the 1A1 cigarette smoke condensate.

CTR-64 To observe the role of particular fractions of the 1A1 condensate on lung tumors induced by MCA.

Objective: Particular fractions of the 1A1 condensate can interrupt AHH activity in vivo. Fractions B_1a (representing 1.0% by weight of the whole condensate) is a potent inducer of pulmonary AHH activity. Fraction SA_W (representing $\approx$ 40% of the total condensate) is a good inhibitor of pulmonary AHH activity. What are the effects of these two fractions on lung tumorigenesis induced by MCA?

Materials:

(1) Mice: C3H/Anf Cum, ♀, 8-10 weeks old

(2) MCA (200μg/0.02ml gelatin solution)

(3) B_1a and SA_W fractions as same concentration ($\approx$ 1000μg/ml) in this same vehicle.

Procedures:

(1) Determine if the B_1a or SA_W fraction will inhibit pulmonary AHH activity when given in same vehicle with MCA. Although the B_1a fraction induces AHH activity it may also inhibit induction by MCA by competative means.

(2) Studies into the effects of these fractions on MCA carcinogenesis will proceed based on these preliminary findings.

SECTION C

CHEMICAL CARCINOGENESIS

C.F. Demoise, Ph.D.

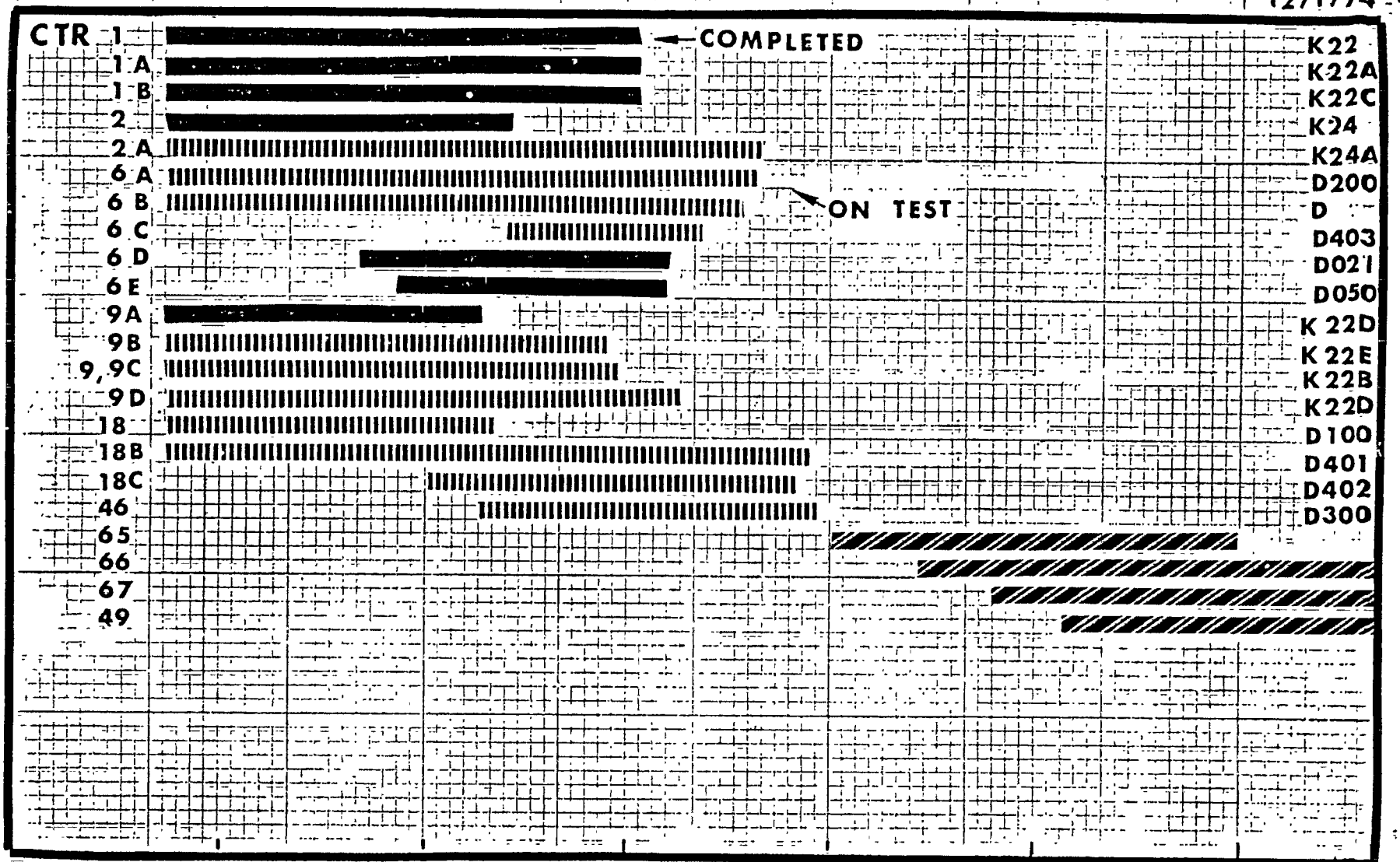
SECTION C SCHEDULE

December 1, 1974

CTR #	MA #	Experiment
CTR-1	(K22)	SC-B:T pellets IRI fractions - MCA cocarcinogenesis.
CTR-1A	(K22A)	SC-B:T pellets IAI fractions - MCA cocarcinogenesis.
CTR-1B	(K22C)	SC-B:T pellets, 15 whole CSC - MCA cocarcinogenesis.
CTR-2	(K24)	IP - Nitrosamine carcinogenesis in C57BL/6 NB mice.
CTR-2A	(K24A)	Repeat of CTR-2.
CTR-6A	(D200)	Immunocompetence - IT-MCA in C3H/f, C57BL/6, and DBA/2 mice.
CTR-6B	(Dseries)	Immunocompetence - It-MCA and DEN in C3H/f mice.
CTR-6C	(D403)	Direct implantation of tumor cells and carcinogens in lung.
CTR-6D	(D021)	Immunocompetence - IT-Fe_2O_3 in C3H/f, C57BL/6 and DBA/2 mice.
CTR-9A	(K22D)	IT-IAI fractions.
CTR-9B	(K22E)	B:T lung implants of 15 CSC samples.
CTR-9 & CTR-9C	(K22B)	IT-15 CSC samples.
CTR-9D	(K22D)	IT-IAI fractions.
CTR-18	(D100)	IT-DEN implant in DBA mice.
CTR-18B	(D401)	B:T lung implants of MCA, BaP, DEN and DMBA in C3H/f, C57BL/6 and DBA/2 mice.
CTR-18C	(D402)	BaP - B:T lung pellet leaching rates.
CTR-46	(D300)	Cell-mediated immune response in mice after SC implantation of wax pellets - MCA.
CTR-65		To determine if immunosuppression plays a role in MCA lung tumor development.
CTR-66		To follow the immunocompetence of C3H/f mice treated by intratracheal instillation of MCA.
CTR-67		To develop an assay for lung carcinogenesis with the nitrosamine compounds.
CTR-49		CSC-IT cocarcinogenesis and/or two-stage carcinogenesis assay system.

SECTION C SCHEDULE

12/1/74



1003336-24

A. New Studies for This Contract Year

1. Bioassay for lung cancer

Preliminary studies carried out during the past year have demonstrated that whole CSC or CSC fractions will not produce lung tumors after 3 injections at a nontoxic dose. It has been shown that SC pellets of CSC or fractions only produce tumors when combined with MCA co-carcinogenesis. It is suggested that the same phenomenon may be true for lung carcinogenesis. Studies are now underway to determine the TuD_{50} via IT inoculation in C3H/f mice for MCA. By combining a subtumorigenic dose of MCA with a nontoxic dose of CSC it may be possible to develop an assay for the lung cancer potential of various tobacco or smog associated chemicals. Such an assay would be more meaningful than the classic skin painting assay now used. It would not only allow the development of a lung tumor assay system but would evaluate this system as an assay procedure for carcinogens normally entering the body by the respiratory tract, but producing tumors elsewhere in the body. This has recently been shown in our laboratory by the IT inoculation of DEN and the development of liver tumors.

CTR-49 December 1, 1974

Objective:

CSC-IT cocarcinogenesis and/or two-stage carcinogenesis assay system.

Procedure:

C3H/Anf Cum and/or B6C3F1 mice will be injected at biweekly intervals for 6-12 injections with subtoxic doses of CSC and CSC fractions combined with 10μg MCA. Mice will be observed for evidence off imminent death before they are sacrificed for gross and histopathological examination.

The use of Fe_2O_3 in combination with the CSC material may prove useful and should be considered since it appears useful in the BaP-Fe_2O_3 experiments.

The vehicle used for suspending the CSC may also be important as shown with the MCA studies; therefore, this aspect will have to be considered in designing these studies.

The use of a promoter as TPA may also prove useful and will be studied if shown useful with low doses of MCA.

2. New Studies to Develop a Model System for Assay of Nitrosamine Carcinogenesis in Mouse Lung Tissues.

During the past several years we have realized the urgent need for consideration of the nitrosamines in lung carcinogenesis. During this period we have been unable to equate the presence of the demethylases to nitrosamine carcinogenesis as we have the AHH system with PAH carcinogenesis. This type of study awaits further definition of this area by Dr. Arcos.

During the new contract year we propose to try a new approach to the study of nitrosamine lung carcinogenesis as outlined briefly in the proposed study.

CTR-67 December 20, 1974

Objective:

To develop an assay for lung carcinogenesis with the nitrosamine compounds.

Background:

The use of IP inoculations of newborn C57BL/6 mice has demonstrated that lung adenomas can be produced in 6-12 months in a limited number of mice. The use of DEN in IT studies failed to produce lung tumors but rather produced liver tumors. Studies are under way using the lung pellet implant procedure with a number of nitrosamines but the assessment of this assay system will require possibly another year.

We propose that a new approach be taken which will insure the assay of nitrosamines for lung tissue will not result in tumors in other tissues by the procedure outlined below.

Procedure:

Mice of several genotypically different backgrounds as the C3H/f, C57BL/6, DBA/2 and possibly others will be anesthetized and the lungs will be infused with various nitrosamine compounds suspended in tissue culture medium believed to be involved in lung carcinogenesis. The lungs will be removed from the animal and held 4 hours in tissue culture medium under the best conditions to allow survival of the cells. The tissue will then be minced and allow time for dialysis of the nitrosamines from the tissues overnight. After this period the tissue will be transplanted either subcutaneously or IP in newborn mice and observed for tumor development. If tumors develop, histological diagnosis of the tumor type will be done to determine if we have, in fact, produced adenomas, adenomocarcinomas or squamous cell carcinomas.

3. New Studies Related to the Immunological Aspects of Chemical Carcinogenesis for the New Contract Year

The studies for the past year have shown some evidence of the suppression effects of chemical carcinogens on the immune competence of the mouse. However, these results have not been as clear cut as we would have hoped them to be. The results of Drs. Richard Levy and Richard Lerner have demonstrated there is probably an association of this ability of the genotypically different strains to metabolize methylcholanthrene (AHH inducibility). Their studies and those carried out in our laboratory by Dr. Demoise have also demonstrated the difficulties in studying cellular immunity in the mouse due to the wide variations in responses after a single intratracheal administration of MCA.

In the new contract year we propose to continue these studies, however to limit this effort and concentrate on an area where we have some evidence of more consistent findings. Our studies have indicated that T and B-cell responses may differ after multiple MCA treatments and during the period when the transformed cells develop into tumors. We feel if we undertake a definitive study with MCA and/or BaP which is paralleled with histological observations and the response to goat RBC by Dr. Levy that we may be able to better define why preliminary results indicate that 6 IT treatments over 12 weeks is superior to 6 treatments over a period of 6 weeks. Another aspect which we feel must be covered by such a study is to determine the kinetics of immune suppression related to ability of spleen cells to respond and the number of cells available for response at varying times after MCA treatment. This may explain differences in in vitro spleen assays and in vivo immune responses which have occurred.

Another aspect of the part immune competence may play in chemical carcinogenesis will also be investigated by immunosuppressing the host in an attempt to shorten the latency period of lung tumor development and possibly decrease the number of treatments required for carcinogenesis.

CTR-65 December 20, 1974

Objective:

To determine if immunosuppression plays a role in MCA lung tumor development.

Background:

Studies carried out under CTR-3 and CTR-7 have demonstrated that the schedule of MCA treatment is possibly more important than the total dose of carcinogen. This may indicate one possible factor at play would be a longer period of immunocompetence with a biweekly schedule vs. a weekly schedule of MCA treatment. If this is true then the period following MCA treatment would be a period when immune competence would be regained and delay tumor development. This also suggests that this hypothesis of immune suppression as a factor in tumor development can be proven if the host is treated with immunosuppressive drugs to allow rapid development of the tumors from cells which were transformed by possibly the first MCA treatment. If we can obtain tumors more rapidly by immunosuppression then one treatment may work as well as multiple carcinogenic exposures.

Procedure:

C3H/f mice will be treated with 250μg MCA at biweekly intervals for 6 treatments. Two weeks after the last treatment the animals will be treated with an immunosuppressive drug cyclophosphamide during the period when tumor development actually occurs. Control animals will be held without immunosuppression to compare the tumor latency period and the number of tumors per animal that develop. The immunosuppressive effect will be determined by tumor transplant or skin graft technique.

If this study shows a shorter latency it will be followed by studies using 1 or 2 MCA treatments followed by immune suppressive therapy.

CTR-66

December 20, 1974

Objective:

To follow the immunocompetence of C3H/f mice treated by intratracheal instillation of MCA in CTR-49.

Procedure:

C3H/f mice will be given 6 or more IT treatments with 250μg MCA at biweekly intervals as described in CTR-49 for histological studies and transplant studies to parallel studies in CTR-6 by Dr. Levy. The spleens and thymuses of these mice will be removed and the average weight and cell ratio determined. These cells will then be used for phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) mitogen in vitro response determination. It is anticipated that multiple doses will induce suppression of cellular response which will probably recover after MCA treatment is stopped. There will probably occur a stimulation of response during the period when tumor growth occurs.

SECTION F. Scientific Data Processing and Analysis

(M.J. Haven, M.S.)

Section F. Scientific Data Processing and Analysis

During the month of October 1974, the entire Microbiological Experiment Management System was transferred from the NIH Computer Center to a commercial facility. In order to transfer and rebuild the Management System, more than 100 data files, software support programs, and the 10,000 records on the data base (which accounts for over 1/2 million fields of experiment information) had to be moved. This transfer was made after an extensive search for a commercial computer that would be compatible with the NIH system as far as software support, costs and file editing capabilities. The transfer necessitated the leasing of an in-house terminal to be used for daily maintenance operations on the data base and periodic updating of current experiments supported by the system. At this time, an average of 5 hours/day is spent on the terminal by MA personnel for the maintenance and expansion of the Experiment Management System.

During the next year, the Scientific Data Processing and Analysis section will be involved with increasing the size capacity for file storage. At present, we can only store information of 30 experiments at one time. We will expand this number to 99 concurrent experiments. Also we now have the capability to store a maximum of 40 observations on each animal for 8 different observation dates during the course of an experiment. We will expand this to 15 different observation dates - thus increasing our total observation number to 115 per animal. We anticipate adding other experiments to the data base within the next 6 months. We are involved in developing summary reports from the data recorded on the autopsy reports that has been entered into the computer. This will add a new dimension in the analysis of the data.

Increased program effort will be focused on the development of support routines for the inhalation experiments. We hope to use new methods and summarization programs in analysing these results. One such method will be the use of a computer generated plotting package. Measures to implement such up-to-date and time saving procedures has already begun. These computer generated graphic plots can be photographed and may accompany any research paper to be published. We also intend to begin the design of an information retrieval system. This fully automated facility will provide the user with a powerfull tool for searching the data base for facts on any experiment. One may thus obtain immediate, organized and complete information on a given subject. This system will be both a research facility and a practical business tool for gathering information at the user's office via a computer terminal on any item of information that has been entered onto the data base.

IV BUDGET FOR CTR-14

JULY 1, 1975 - JUNE 30, 1976

10035736734

IV BUDGET

The budget has been presented this year based on individual budgets for each section within this contract. Each scientific investigator has two technicians and the necessary animal caretaker support. Two histology laboratory sections (Sections D and E) are included. The services of the slide preparation laboratory (D) will be phased out during the first quarter of the year and all slides will be made in Section E. Dr. B. Sass (Section D) will continue to service this contract as his experience is invaluable. The Data Processing and Analysis Section (F) has become a very valuable part of our contract making available analysis of our data in greater detail than possible by manual operations.

We feel the budget presented here represents the lowest possible level to present an integrated, yet diversified program which is outlined in the proposal. The inflationary pressures have been exceedingly great during the past year and the future appears to promise even greater stress in this area. We have included funds for equipment which has been shared by other contracts in the past and has been supplemented on a short term basis with equipment to be used in the Inhalation Laboratory the next contract year. We feel only by going to permanent cages can we continue to operate an animal facility at a reasonable price.

BUDGET FOR CTR-14 (July 1, 1975 - June 30, 1976)
(MA 1800-2222)

		Section of Contract						
		A*	B	C	D	E	F	Total
A.	Direct Labor (Schedule A)	$ 40,144	$ 27,026	$ 35,215	$ 8,794	$ 11,644	$ 10,075	$132,898
B.	Overhead (115% of A)	46,166	31,080	40,497	10,113	13,391	11,586	152,833
C.	Other Direct Costs	22,000	18,000	14,000	1,000	3,000	10,000	68,000
D.	Travel	1,500	500	1,000	500	--	700	4,200
E.	Total (A-D)	109,810	76,606	90,712	20,407	28,035	32,361	357,931
F.	G & A (16%)	17,570	12,257	14,514	3,265	4,485	5,178	57,269
G.	Overtime Premium	200	200	200	--	--	--	600
H.	Total Cost	127,580	89,063	105,425	23,672	32,521	37,539	415,800
I.	Fixed Fee	14,174	9,895	11,713	3,628	3,613	4,171	46,194
J.	Total Before Equipment	$141,754	$ 98,958	$117,138	$ 26,300	$ 36,134	$ 41,710	$461,994
K.	Equipment (Schedule B)							60,800
L.	Total							$522,794

*A. C.E. Whitmire, Chemical Carcinogenesis
B. R.E. Kouri, AHH-Related Chemical Carcinogenesis
C. C.F. Demoise, Chemical Carcinogenesis
D. B. Sass, Histology at Walkersville during transition period
E. Histology in Bethesda Laboratory
F. M. Haven, Data Processing and Analysis

100353736

SCHEDULE A - Direct Labor for CTR-14 (MA 2222)
(July 1, 1975 - June 30, 1976)

Section/Personnel	$/Hr.	% Time	Hours	$	$
A. Chemical Carcinogenesis					
C.E. Whitmire, Ph.D.	REDACTED	50	963	REDACTED	
R.M. Mason, Sr. Tech.		100	1,926		
P.G. Stein, Tech.		100	1,926		
A. Zuna, Anim. Care.		100	1,926		
			6,741		
			R	2,626	40,144
B. AHH-Related Chem. Carcinogenesis					
R.E. Kouri, Ph.D.	REDACTED	10	193	REDACTED	
T. Rude, Tech.		100	1,926		
E. Kiss, Tech.		100	1,926		
E. Prieto, Jr. Tech.		50	963		
			5,008		
			R	1,768	27,026
C. Chemical Carcinogenesis					
C.F. Demoise, Ph.D.	REDACTED	75	1,445	REDACTED	
M.D. Avery, Tech.		100	1,926		
K. Thorson, Tech.		100	1,926		
H. Lopez, Anim. Care.		100	1,926		
			7,223		
			R	2,304	35,215
D. Histopathology-Walkersville					
B. Sass, D.V.M.		25	482		
F. Dorset, Tech.	REDACTED	25	482	REDACTED	
B. Fouch, Tech.		25	482		
			1,446		
			R	575	8,794
E. Histology-Bethesda					
S.A. Gosnell, Super. Tech.	REDACTED	50	963	R	
Vacancy, Tech.		100	1,926		
			2,889		
			R	762	11,644
F. Data Processing and Analysis					
M. Haven, M.S.	REDACTED	10	196		
P. Gradwell, Data Tech.		55	1,059	REDACTED	
B. Ross, Key Punch Tech.		50	963		
			2,218		
			R	659	10,075
			25,525 hrs. (13.24 man years)		$132,898

1003536737

Schedule B: CTR-14 (July 1, 1975 - June 30, 1976)

During the past years of this contract, CTR has operated in a facility shared by an NCI contract and has made use of the equipment of that contract. Due to the size of this contract, it has been essential to provide separate facilities, therefore there exist a shortage of essential equipment for an efficient operation. During the past year the equipment destined for CTR-22 inhalation laboratory has been utilized for CTR-14. When the new facility for CTR-22 is completed, this equipment must be moved, thus leaving CTR-14 without essential equipment. The following list is submitted of essential items to maintain the status quo in our operation:

CO_2 incubator	$ 3,000
Ice machine	650
Nitrogen freezer with inventory system	6,000
Centrifuge	4,000
Balance	1,000
Rotary apparatus	350
2 water baths	800
Automatic watering on existing racks	15,000
Permanent animal cages	30,000
	$60,800

CURRICULUM VITAE FOR NEW PERSONNEL

CURRICULUM VITAE - MILES J. HAVEN

BIRTH: R

EDUCATION:

1972 M.S. Computer Science
University of Maryland, College Park

1967 B.S. Computer Science
University of Maryland, College Park

PROFESSIONAL AFFILIATIONS: REDACTED

PRESENT POSITION: September, 1974 - present REDACTED

POSITION DESCRIPTION:

REDACTED

PRIOR EXPERIENCE:

1971 - 1974

1967 - 1971 REDACTED

1968 - present

10035367740

PUBLICATIONS - MILES J. HAVEN

Glaser, E., and Haven, M. Band Pass Noise Stimulation of the Simulated Basilar Membrane. J. of Acoustical Society of America, October 1972.

Haven, M. Simulation and Mbdel Building. M.S. Thesis, University of Maryland, January 1972.

VI PUBLICATIONS

VII. PUBLICATIONS - 1974

Whitmire, C.E., Demoise, C.F., Kouri, R.E. The Role of the Host in the Development of In Vivo Models for Carcinogenesis Studies. In: Symposium on Experimental Respiratory Carcinogenesis and Bioassays (ed. J.F. PARKS and E. KARBE) Springer-Verlag, 1974, pages 20-47.

Demoise, C.F., Kouri, R.E., and Whitmire, C.E. Cell-mediated Immunity After Intratracheal Exposure to 3-Methylcholanthrene, and its Relationship to Tumor Transplant Growth in C3H/fMai Mice. In: Symposium on Experimental Respiratory Carcinogenesis and Bioassays (ed. J.F. PARKS and E. KARBE) Springer-Verlag, 1974, pages 72-80.

Kouri, R.E., Demoise, C.F., Whitmire, C.E. The Significance of the Aryl Hydrocarbon Hydroxylase Enzyme Systems in the Selection of Model Systems for Respiratory Carcinogenesis. In: Symposium on Experimental Respiratory Carcinogenesis and Bioassays (ed. J.F. PARKS and E. KARBE) Springer-Verlag, 1974, pages 48-61.

Kouri, R.E., Ratrie III, H., Whitmire, C.E. Genetic Control of Susceptibility to 3-Methylcholanthrene-Induced Subcutaneous Sarcomas. Int. J. Cancer 13: 714-720, 1974.

Kouri, R.E., Kiefer, R., Zimmerman, E.M. Hydrocarbon-Metabolizing Activity of Various Mammalian Cells in Culture. In Vitro, Vol. 10, pages 18-25, 1974.

Kouri, R., Ratrie III, H., Atlas, S.A., Niwa, A., Nebert, D.W. Aryl Hydrocarbon Hydroxylase Induction in Human Lymphocyte Cultures by 2,3,7,8-Tetrachlorodibenzo-p-dioxin. Life Sciences, vol. 15, pages 1585-1595, 1974.

Benedict, W.F., Rucker, N., Mark, C., Kouri, R.E. Correlation Between the Balance of Specific Chromosomes and the Expression of Malignancy in Hamster Cells. J. Natl. Cancer Inst. (in press) 1975.

Kouri, R.E., Rude, T.H., Thomas, P.E., Whitmire, C.E. Studies on Pulmonary Aryl Hydrocarbon Hydroxylase in Inbred Strains of Mice. (submitted) 1974.

Kouri, R.E., Kurtz, S.A., Price, P.J., Benedict, W.F. Studies on the ara-C-Induced Malignant Transformation of Hamster and Rat Cells in Culture. (submitted) 1974.

Kouri, R.E. Genetic Control of Susceptibility to Cancer Induced by 3-Methylcholanthrene (MCA). Proceedings of the XI International Cancer Congress, October 1974.

Benedict, W.F., Rucker, N., Faust, J. and Kouri, R.E. Malignant Transformation of Mouse Cells by Cigarette Smoke Condensate. Journal of the National Cancer Institute (in press) 1975.

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The Significance of Aryl Hydrocarbon Hydroxylase Enzyme Systems in the Selection of Model Systems for Respiratory Carcinogenesis*

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Department of Experimental Oncology, Viral-Chemical Carcinogenesis Section, Bethesda, MD 20014, USA

ABSTRACT

Aryl hydrocarbon hydroxylase (AHH) is a multicomponent, microsomal-bound enzyme system which converts a variety of lipid-soluble compounds to water-soluble forms for subsequent elimination from the body. The enzyme system is inducible by a variety of endogenous and exogenous compounds including steroid hormones, barbiturates, insecticides, polycyclic aromatic hydrocarbons (PAH), and whole cigarette smoke. Recent results have demonstrated that inducibility is host-gene regulated, the inducibility of this enzyme correlates with carcinogenic susceptibility to PAH in animal, and bronchogenic squamous cell carcinoma in humans (probably cigarette smoke induced).

This paper illustrates the types of AHH responses observed in pulmonary tissues following treatment of mice of various strains with either PAH or tobacco related chemicals. Following intratracheal instillation of 3-methylcholanthrene, we observed that: a) pulmonary AHH can be induced preferentially at doses <200 µg (in contrast to higher doses that induce both hepatic and pulmonary tissues), b) kinetic data demonstrate a 6 to 8 fold increase within 24 hours followed by a broad plateau lasting up to 96 hours, and c) induction is host regulated, segregating as a single autosomal dominant gene in crosses between the C57BL/6 (inducible) and DBA/2 (noninducible) strains of mice. Although DBA/2 pulmonary tissue is slightly inducible (in contrast to the noninducibility of hepatic tissue), evidence indicates that this response results from proliferation of constitutive AHH and not true "induction". Exposure to whole smoke from one 1A1 cigarette (10% smoke in a Walton Horizontal Smoking Machine) will preferentially induce pulmonary AHH, and this response is under the same genetic control as that induced by MCA. Exposure to gas phase alone will not induce this response. Use of cigarette smoke condensate fractions (Stedman fractionation) derived from 1A1 tobacco show that after intratracheal instillation, at least 4 fractions are capable of inducing pulmonary AHH. Fractions 3, 4, 12, and 14 (Bi^a, Bi^b, N_{NM}, and N_{MEOH}) induce at least a 2 fold increase of pulmonary AHH (at a LD_{10-14} dose). It seems as if the enzymatic potential of the lung tissue itself may be a major determinant in the ultimate fate of this organ in any carcinogenic process.

*This research was supported by the Council for Tobacco Research.

A. Introduction

Aryl hydrocarbon hydroxylase (AHH) is the name given to one of the multi-component, mixed-function oxidases that converts a variety of lipid-soluble endogenous and exogenous compounds to water-soluble forms, usually for subsequent elimination from the body (MASON, 1957; CONNEY, 1967). The enzyme system possesses 2 properties which make it particularly amenable for studying its role in chemically induced cancers: 1. the system is inducible**(NEBERT and GELBOIN, 1969) and 2. this inducibility is regulated by a single autosomal dominant gene in crosses involving the C57BL/6 and DBA/2 strains of mice (THOMAS et al., 1972; NEBERT et al., 1972). Recent information suggests that this enzyme system plays a major role in 3-methylcholanthrene-induced carcinogenesis in the aforementioned mouse strains (KOURI et al., 1973a, 1973b, 1974a).

The lung and skin of mice seem to be under a different type of genetic control from that of hepatic tissue, because these organs appear to be slightly inducible in strains in which the liver is completely non-inducible (BURKI et al., 1973; WIEBEL et al., 1973). There are many questions concerning the pulmonary response: Is there really a separate genetic control for lung AHH levels? Do genetically regulated differences in AHH inducibility exist in pulmonary tissue of inbred mice? What is the effect of other exogenous chemicals (e.g., tobacco-related products) on lung AHH? Do these enzymatic responses play a role in the susceptibility of mice to chemically induced lung cancers? In this report, we attempt to answer some of the questions concerning the pulmonary response.

B. Materials

The polycyclic hydrocarbons benzo(a)pyrene (BaP) and 3-methylcholanthrene (MCA) were purchased (Sigma Chemicals, St. Louis, Missouri) and purified by recrystallization from benzene. 7,8-benzoflavone was purchased from Aldrich Chemicals (Cedar Knolls, New Jersey). Sources of mice were Cumberland View Farms (Clinton, Tennessee), the Jackson Laboratory (Bar Harbor, Maine), or Microbiological Associates (Bethesda, Maryland). For intratracheal (IT) instillations, a Bausch and Lomb stereomicroscope, equipped with fiber optic illumination, and Hamilton syringes with 22 gauge by 38 mm (with 1.5 mm feeder balls) needles were used. Fractions of cigarette smoke condensate from 1A1 cigarettes were provided by Dr. A.R. PATEL (Meloy Laboratories, Springfield, Virginia) (PATEL et al., 1974). Fractionation into acidic, basic, and neutral fractions was performed according to the procedures of SWAIN et al. (1969). Only 3 fractions have been analyzed as to chemical content: the B_E fraction contains 310 mg nicotine per g fraction, the WA_E fraction contains 41.9 mg phenols per g fraction, and the N_{NM} fraction contains 13.2 µg BP per g fraction (PATEL et al., 1974). Walton-type horizontal smoking machines were obtained from Process and Instruments (Brooklyn, New York), cigarettes were either the 1A1 or 1R1 type (University of Kentucky, Lexington). Enzyme determinations were made

** The term "inducibility", as used in this paper, denotes a relative increase in rates of de novo synthesis or of activation of enzyme activity from preexisting moieties, or in rate of both when compared to rate of breakdown. No particular mechanism is implied.

using an Aminco-Bowman spectrophotofluorometer (American Instrument Company, Silver Spring, Maryland).

C. Methods

Care and feeding of mice were as previously published (WHITMIRE et al., 1971). Animals were always treated between the hours of 9:00 am and 10:00 am to avoid diurnal variations. The intratracheal instillation technique was similar to that described recently by HO and FURST (1973). Solutions consisted of MCA suspended in 0.2% gelatin in sterile saline or the various cigarette smoke condensate fractions dissolved in corn oil. Condensate fractions were used at an arbitrary level that killed 10% of the mice in 14 days (LD_{10-14}). 0.02 ml of solution was instilled. At various times post-treatment, lungs and livers were excized and frozen at -70°C until assayed.

Microsomes were prepared from liver tissues of mice pretreated with MCA according to the methods of KUPFER and LEVIN (1972) and were stored at -70°C for up to 72 hours before being assayed. Calcium-aggregated and "normal" centrifugally prepared microsomes were used with similar results. Samples were diluted with 0.1M tris-HCl buffer (pH 7.4) to a final ratio of 1.0 ml microsome suspension per wet weight tissue.

The assay for AHH was basically that of NEBERT and GELBOIN (1969), as modified by NEBERT and GIELEN (1972) and THOMAS et al. (1972). A unit of AHH activity is that amount of enzyme causing the fluorescent equivalent of 1.0nMole 3-OHBP per min at 37°C. For hepatic and pulmonary tissues, activity is given in terms of units/g wet weight tissue and for microsome preparations in terms of units/mg protein.

Inhibition of BaP metabolism *in vitro* was done according to the procedures of GOUJON et al. (1972) and WIEBEL et al. (1971). Concentrations of the various condensate fractions were made in dimethylsulfoxide (DMSO); included was the known competitive inhibitor of "induced" AHH activity, 7,8-benzoflavone (WIEBEL et al., 1971). 200 µg, 20 µg, and 2 µg of the fractions were added to the complete reaction mixture (minus BaP), incubated with shaking for 1.0 min, and then BaP (20 µg) was added and incubation continued for 20 min.

Mice were exposed to 1, 2, or 3 cigarettes simultaneously (10%, 20%, or 30% smoke) and were also exposed to the smoke of 1 cigarette (10% smoke) for 1.0 hour (about 7 cigarettes). This latter exposure regimen consisted of exposing mice to 1 cigarette followed by a 10 min rest period followed by 1 cigarette, until a total of 7 cigarettes were smoked. In some cases, mice were exposed to cigarette smoke for at least 60 days at 8 cigarettes per day (4 consecutive cigarettes in morning and 4 in afternoon). At various times after exposure, mice were killed by cervical dislocation and the lungs and livers were removed and stored at -70°C until assayed. Control animals consisted of either untreated, sham-smoked, or gas-phase (Cambridge-filtered smoke) treated animals. Mice were exposed in 1 min cycles consisting of a 2 sec puff, 15 sec holding time, and 43 sec purge. In certain experiments, holding time was increased to 28 sec and purge time was 30 sec.

D. Results

I. Pulmonary and Hepatic AHH Responses to IT Instilled MCA

The pulmonary and hepatic AHH levels 24 hours after IT administration of various dose of MCA into C57BL/6Cum (B6) DBA/2Cum (D2) and B6D2F1 mice are shown in Table 1. At doses greater than 200 µg, the AHH responses of pulmonary tissues from B6 and B6D2F1 lungs were maximally induced (about 7 fold) while hepatic tissues were only minimally effected. The D2 strain was generally much less responsive: pulmonary tissue was only minimally induced at a dose of 500 µg and hepatic tissue was never induced, regardless of MCA dose. Kinetics of induction of pulmonary AHH in these 3 strains following IT treatment with 200 µg MCA are shown in Fig. 1. Maximum induction in the B6 and B6D2F1 strains occurred by 24 hours and remained constant for at least 96 hours. The D2 strain was observed to respond slowly to MCA and maximal induction occurred 48 hours posttreatment. The maximum observed increase (inducibility) for the B6 or B6D2F1 strains was about 10 and for the D2 strain about 6.

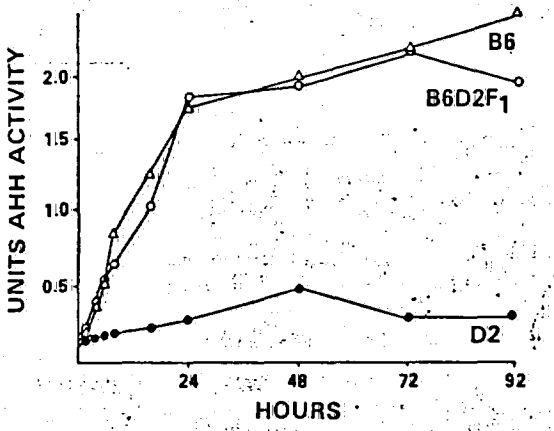

Fig. 1

Responses of 8 other inbred strains to 200 µg MCA given IT are shown in Table 2. Pulmonary tissues of BALB/cMai C3H/fMai, C57L/J, and C57BL/6J were observed to be highly induced by 200 µg MCA (4 to 8 fold), while lung tissue from strains AKR/J, SJL/J, DBA/2J, and RF/J showed no such increase. Hepatic responses were low for all strains except perhaps for the C57BL/6J and C57L/J, which did express a 1-fold increase..

II. Genetic Regulation of Pulmonary AHH Induction

The effect of MCA on pulmonary tissue from crosses involving the B6 and D2 strains is shown in Table 3. Animals were classified as inducible or noninducible if, after IT treatment with 200 µg MCA, pulmonary AHH levels were 2.5 (± 0.3) units/g tissue (inducible) or 0.3 (± 0.05) units/g tissue (noninducible). Among 47 backcross animals

Table 1. Effects of intratracheal instillation of various doses of MCA in a 0.2% gelatin solution on pulmonary and hepatic AHH[a] levels

STRAIN AND TISSUE	UNTREATED	GEL	MCA 10 µg	MCA 50 µg	MCA 200 µg	MCA 500 µg
C57BL/6Cum						
LUNG	0.40	0.32	0.89 (2.8)[b]	1.4 (4.4)	2.4 (7.5)	2.4 (7.5)
LIVER	17.50	14.50	13.30 (0.9)	14.51 (1.0)	32.3 (2.2)	33.2 (2.3)
DBA/2Cum						
LUNG	0.30	0.18	—	0.20 (1.1)	0.34 (1.9)	0.56 (3.1)
LIVER	9.80	9.50	—	9.80 (1.0)	10.10 (1.1)	10.20 (1.1)
B6D2F1Cum						
LUNG	0.36	0.26	0.76 (2.9)	1.3 (5.0)	2.0 (7.7)	2.5 (9.6)
LIVER	12.60	10.00	10.30 (1.0)	14.4 (1.4)	18.9 (1.9)	26.5 (2.7)

[a]AHH ACTIVITY GIVEN IN TERMS OF UNITS PER g WET WEIGHT TISSUE. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1.0 nmole 3-OH-BP PER MINUTE AT 37°C

[b]THE INDUCIBILITY (A RELATIVE INCREASE OF AHH OF TREATED TISSUE COMPARED TO CONTROL TISSUE) IS GIVEN PARENTHETICALLY

Table 2. Effects of intratracheal instillation of 200 µg MCA in 0.2% gelatin on pulmonary and hepatic AHH[a] in various strains of mice

	LUNG AHH			LIVER AHH		
STRAIN	CONTROL	MCA	IND.[b]	CONTROL	MCA	IND.
Balb/cMai	0.71	3.1	4.4	19.2	22.2	1.2
C3H/fMai	0.33	2.5	7.7	7.9	7.3	0.93
C57L/J	0.64	3.4	5.3	13.3	27.4	2.1
C57BL/6J	0.28	2.4	8.0	16.1	32.6	2.0
AKR/J	0.28	0.45	1.6	17.5	15.6	0.89
SJL/J	0.19	0.29	1.5	10.8	11.7	1.1
DBA/2J	0.26	0.36	1.4	8.9	8.5	0.95
RF/J	0.41	0.54	1.3	13.5	13.8	1.0

[a]AHH ACTIVITY GIVEN IN TERMS OF UNITS PER g WET WEIGHT TISSUES. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1.0 nmole OF 3-OH-BP PER MINUTE AT 37°C

[b]IND. = INDUCIBILITY; RELATIVE INCREASE OF AHH OF TREATED TISSUE COMPARED TO CONTROL TISSUE

tested, 24 were inducible (51%), and among 42-F2 animals tested, 29 were inducible (69%). These numbers were not statistically different from the 50% and 75% ratios that would be expected if a single autosomal dominant gene were regulating this inducibility.

Table 3. Genetic regulation of pulmonary AHH in crosses involving the C57BL/6Cum and DBA/2Cum strains of mice[a]

STRAIN	NUMBER OF MICE TREATED	NUMBER OF MICE INDUCIBLE	%
B6	50	50	100
D2	50	0	0
B6D2F1	50	50	100
B6D2F1 x D2	47	24	51
B6D2F2	42	29	69

[a]MICE WERE TREATED WITH 200 µg MCA/.02 ml-0.2% GELATIN SOLUTION INTRATRACHEALLY, AND 24 HOURS LATER, THE PULMONARY AHH WAS ASSAYED. A MOUSE WAS CONSIDERED INDUCIBLE IF, AFTER MCA TREATMENT, PULMONARY AHH WAS 2.5 (+ 0.3) UNITS/g TISSUE AND CONSIDERED NONINDUCIBLE IF PULMONARY AHH WAS 0.30 (+ 0.05) UNITS/g TISSUE. THE SEX OF THE PROGENY PLAYED NO ROLE IN THIS SEGREGATION PATTERN.

III. Effect of Tobacco Related Products on Pulmonary AHH Levels

1. Effect of Cigarette Smoke. The pulmonary AHH response of B6 mice exposed to the smoke of one-1A1 cigarette (10% smoke) is shown in Table 4. The lung tissue responded rapidly and selectively to the whole smoke. Peak activity occurred approximately 6 hours posttreatment and remained induced for 24 hours. The intervention of a Cambridge-type filter completely abrogated this induction profile. The use of 2 or 3 cigarettes smoked simultaneously (20% or 30% smoke) gave induction results similar to the use of 1 cigarette (data not shown). Exposure to 1 cigarette-at-a-time for a total of 7 cigarettes (with a 10 min rest between cigarettes) induced pulmonary AHH activity in B6 and C3H/fMai mice, but had only a small effect on D2 mice (Table 5). This smoking schedule resulted in maximal exposure with minimal death if nonpretreated animals were used. Using this schedule, the maximal induction was similar to that induced by 1 cigarette (Table 4): about 2.5 fold in 6 hours. Similar results were also noted using the 1R1 cigarette, i.e., maximal induction occurred within 6 hours after exposure and induction was about 2.5 times that of the sham control or gas-phase treated animals. Similar results were also noted if the holding time was increased from 15 to 28 sec. The 28 sec holding time should allow for maximum deposition of particulate material onto lung tissue (STOCKLEY, Oak Ridge National Laboratory, personal communication, 1974).

Mice could be adapted to higher smoke exposures by pretreatment with only 1 or 2 cigarettes per day for 1 week. By slowly increasing the number of cigarettes (given 1-at-a-time) per day, at 1 month, both D2 and C3H/fMai mice would accept 16 cigarettes per day, 8 consecutive cigarettes in the morning and 8 consecutive in the afternoon. Although slightly more toxic initially, mice would still accept 16-1R1 cigarettes per day using this same schedule. The AHH responses of various tissues of these 2 strains after exposure to 8 cigarettes per day (4 in the morning and 4 in the afternoon) for at least 60 days are demonstrated in Table 6. The chronic high level of smoke seemed to induce only pulmonary tissue; liver, kidney, and small intestinal tissue was unaffected. The data with the intestines was difficult to assess because of the wide mouse-to-mouse variations observed (>200%). Pulmonary tissue from C3H/fMai mice was induced for the whole 18 hours observation period, while the induction of D2 lung tissue seemed to

Table 4. Effect of exposure to one 1A1 cigarette (10% smoke) on pulmonary AHH levels of C57BL/6Cum mice[a]

	AHH ACTIVITY (UNITS[b]/g TISSUE)		
HR. AFTER SMOKE	WITH FILTER	WITHOUT FILTER	INDUCIBILITY
1.5	0.25	0.24	1.0
3.5	0.21	0.43	1.8
6.5	0.31	0.86	3.6
9.0	0.25	0.53	2.2
12.0	0.25	0.58	2.4
27.0	0.27	0.55	2.3
50.0	0.27	0.38	1.6
74.0	0.27	0.38	1.6
CONTROL	0.24	0.24	

[a]MICE WERE EXPOSED IN A WALTON-TYPE HORIZONTAL SMOKING MACHINE ACCORDING TO THE FOLLOWING 1 MIN CYCLE: 2 SEC PUFF, 15 SEC HOLDING TIME AND 45 SEC PURGE

[b]UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP/min AT 37°C

Table 5. Effect of 7 consecutive cigarettes[a] on pulmonary AHH activity in various strains of mice

		HOURS[b] POST TREATMENT			
		6		24	
STRAIN	CONTROL	SMOKED	INDUCIBILITY	SMOKED	INDUCIBILITY
C57BL/6Cum	0.20[c]	0.50	2.5	0.45	2.3
DBA/2J	0.25	0.34	1.4	0.32	1.4
C3H/fMai	0.34	0.88	2.6	0.65	1.9

[a]ANIMALS WERE EXPOSED TO CIGARETTE SMOKE USING THE REGIMEN OF ONE CIGARETTE FOLLOWED BY A 10 MIN REST PERIOD FOLLOWED BY ONE CIGARETTE, UNTIL A TOTAL OF SEVEN CIGARETTES WERE SMOKED

[b]HOURS AFTER EXPOSURE TO LAST OF SEVEN (7) 1A1 CIGARETTES

[c]DATA GIVEN IN TERMS OF UNITS AHH ACTIVITY PER g TISSUE. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP PER MIN AT 37°C

have a shorter lifetime and was at background level by 18 hours post-treatment. Responses to the 1R1 cigarettes was similar to that observed for the 1A1 cigarettes.

2. Effect of Cigarette Smoke Condensate Fractions. Fractions of 1A1 cigarette smoke condensate were observed to induce and to inhibit the pulmonary AHH activity of B6 mice 24 hours after IT instillation (Table 7). Fractions $B_I a$, $B_I b$, N_{MEOH}, and N_{NM} were considered good inducers. The starting material, reconstituted material, B_E, WA_E, WA_I, and N_{CH} were considered weak inducers. Fractions B_W, SA_I, SA_E, and SA_W were actually weak inhibitors of pulmonary AHH activity.

Particular fractions also seemed to have the capability of inhibiting BaP metabolism *in vitro* (Table 8). Data is presented by computing the

Table 6. Effect of smoking[a] on AHH responses of various tissues of 2 inbred strains of mice

		HOURS POST TREATMENT								
		3			6			18		
STRAIN	TISSUE	CONTROL	SMOKED	IND.[b]	CONTROL	SMOKED	IND.[b]	CONTROL	SMOKED	IND.[b]
C3H	LUNG	0.34	1.01	3.1	0.48	0.95	2.0	0.23	0.51	2.2
	LIVER	9.90	10.31	1.1	8.00	9.40	1.2	11.00	13.70	1.2
	KIDNEY	0.06	0.06	1.0	0.07	0.06	0.9	0.06	0.07	1.1
	INTESTINE	1.10	0.63	0.6	0.28	0.30	1.1	0.25	0.35	1.4
D2	LUNG	0.36	0.82	2.3	0.35	1.00	2.9	0.30	0.30	1.0
	LIVER	8.00	10.50	1.3	10.90	9.50	0.9	13.50	14.00	1.0
	KIDNEY	0.08	0.07	0.9	0.06	0.06	1.0	0.07	0.07	1.0
	INTESTINE	0.28	0.99	3.5	0.07	0.08	1.1	0.09	0.07	0.8

[a]ANIMALS WERE PRESMOKED FOR AT LEAST 60 DAYS BY EXPOSURE TO FOUR (4) CONSECUTIVE CIGARETTES GIVEN IN THE MORNING AND FOUR (4) CONSECUTIVE CIGARETTES GIVEN IN THE AFTERNOON. INDICATED TIMES ARE HOURS AFTER EXPOSURE TO LAST OF THE 4 CONSECUTIVE CIGARETTES. CONTROL ANIMALS WERE UNTREATED

[b]IND. = INDUCIBILITY

Table 7. Effect of fractions of the 1A1 cigarette-smoke-condensate on pulmonary AHH activity of C57BL/6Cum mice[a]

FRACTION NO.[b]	FRACTION	µg	UNITS/g TISSUE[c]	INDUCIBILITY
1	STARTING MATERIAL	2000	.22	1.7
2	RECONSTITUTED	500	.24	1.8
3	$B_I a$	1000	.47	3.6
4	$B_I b$	1000	.32	2.5
5	B_E	50	.19	1.5
6	B_W	500	.07	0.5
7	WA_I	1000	.21	1.6
8	WA_E	500	.24	1.1
9	SA_I	500	.07	0.5
10	SA_E	500	.04	0.3
11	SA_W	2000	.05	0.4
12	N_{MEOH}	2000	.32	2.5
13	N_{CH}	500	.15	1.2
14	N_{NM}	500	.43	3.3
CONTROL	CORN OIL	–	.13	1.0

[a]24 HOURS AFTER IT INSTILLATION OF FRACTION, OR CORN OIL VEHICLE

[b]ARRANGED ACCORDING TO SWAIN et al (1969)

[c]A UNIT OF AHH ACTIVITY IS THAT AMOUNT OF ENZYME CAUSING THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP/min AT 37°C

Table 8. *In vitro* effect of cigarette smoke condensate fractions on BaP metabolism in hepatic microsomes[a] from MCA treated B6 mice

FRACTION		[X]/[BaP] TO GIVE 50% INHIBITION[b]
1	STARTING MATERIAL	5.0
2	RECONSTITUTED	5.2
3	$B_I a$	0.8
4	$B_I b$	0.5
5	B_E	3.0
6	B_W	>10.
7	WA_I	5.0
8	WA_E	2.0
9	SA_I	>10.
10	SA_E	>10.
11	SA_W	>10.
12	N_{MEOH}	3.0
13	N_{CH}	ND
14	N_{NM}	1.0
–	7, 8-BENZOFLAVONE	1.0

[a]SOURCE OF MICROSOMES WAS HEPATIC TISSUE FROM MICE PRETREATED 24 HOURS PREVIOUS TO SACRIFICED WITH 80 µg MCA/g BODY WEIGHT GIVEN INTRAPERITONEALLY. SPECIFIC ACTIVITY OF MICROSOMES WAS 0.595 units/mg PROTEIN AND DMSO TREATED CONTROL MICROSOMES WAS 0.583 units/mg PROTEIN

[b]200, 20 and 2 µg OF THE VARIOUS FRACTIONS WERE ADDED TO THE COMPLETE REACTION MIXTURE (EXCEPT BaP), INCUBATED WITH SHAKING AT 37°C FOR 1.0 MIN., AND THEN BaP (20 µg) WAS ADDED AND INCUBATION CONTINUED FOR 20 MIN. DATA GIVEN IN TERMS OF CONCENTRATION OF BaP REQUIRED TO INHIBIT THE FORMATION OF 3-OH-BP BY 50%

concentration of the material over the concentration of BaP necessary to inhibit enzyme activity by 50% (GOUJON et al., 1973). Using a microsomal preparation from MCA-induced livers, fractions $B_I b$, $B_I a$, and N_{NM} inhibited BaP metabolism at least as effectively as the known competitive inhibitor of induced AHH, 7,8-benzoflavone. The starting material, reconstituted material, B_E, WA_I, WA_E, N_{MEOH}, and N_{CH} fractions were weak inhibitors, while the B_W, SA_I, SA_E, and SA_W fractions had no effect on BaP metabolism.

E. Discussion

There are major strain-to-strain variations in the AHH levels of inbred strains of mice (THOMAS et al., 1972). Similar variations in AHH activity (or inducibility) also exist in the human system (KELLERMANN et al., 1973a). These variations are under host genetic control in both mice and man, segregating as either a dominant, codominant, or recessive gene in mice, depending on strains employed (ROBINSON et al., 1974), and a single codominant gene in man (KELLERMANN et al., 1973b). Recent information suggests that sensitivity or susceptibility to chemically induced cancers is correlated with the AHH responsiveness of that individual. Individual mice or strains of mice which are AHH inducible are much more sensitive to MCA induced tumors than their noninducible counterparts (KOURI et al., 1973a, 1973b, 1974a); and, in man, individuals with high AHH inducibilities seem much more sensitive to cigarette smoke associated bronchogenic squamous cell carcinomas (KELLERMANN et al., 1973c). In the mouse, hepatic tissue has been used to determine the AHH inducibility of individuals. A valid question would be, does the liver activate (or inactivate) carcinogens for other tissues, or can other organs determine their own sensitivity to chemical carcinogens? Results presented here suggest that at least one other organ, the lung, can be a major determinant in its own ultimate susceptibility to chemically induced cancers.

Using the intratracheal route (to limit the enzymatic response to pulmonary tissue alone) and the B6 and D2 inbred strains of mice whose hepatic AHH responses have been extensively studied, we show in this report: 1. MCA given in a 0.2% gelatin solution induces pulmonary AHH, and this induction is dose dependent; 2. a dose of 200 µg MCA maximally induces pulmonary AHH, but has very limited effect on hepatic AHH levels; 3. pulmonary AHH can be induced in D2 mice by IT administration of MCA, but hepatic AHH levels are never induced; 4. although pulmonary AHH is induced in D2 mice, the levels are very low, with about a 5 fold difference between D2 and B6 pulmonary tissues; and 5. this strain difference is under the same genetic control as that of hepatic tissue, e.g., the highly responsive B6 strain differs from the D2 strain by a single autosomal dominant gene controlling this heightened responsiveness. Results with other inbred strains of mice agree with this contention: strains that are nonresponsive to MCA in their hepatic tissues (THOMAS et al., 1972) are low responders in their lung tissue to IT instilled MCA and vice versa.

These results may seem inconsistent in that the pulmonary tissue of both B6 and D2 mice are induced by IT administration of MCA, agreeing with the results of WIEBEL et al. (1973) and BURKI et al. (1973), yet there seem to be basic differences between these 2 "induction" processes because the responses can be discriminated genetically (Table 3). Recent results from our laboratory (KOURI et al., 1974b) suggest that there is, in fact, a difference between the "induction" of pulmonary tissues of the B6 and D2 strains. Use of the competitive

inhibitor of induced AHH, 7,8-benzoflavone, and direct quantification of the CO-binding cytochromes from B6 and D2 lung tissue demonstrate that real differences exist between the enzymes in MCA treated B6 and D2 pulmonary tissue. Available data are consistent with the hypothesis that MCA treatment of B6 lung tissue induces the genetically mediated AHH enzyme system associated with the P-448 cytochrome (SLADEK and MANNERING, 1966), and treatment of D2 lung tissue causes the nonspecific proliferation of enzymes that are very similar to the constitutive, or P-450 mediated (GILLETTE et al., 1972), enzymes. Thus, pulmonary AHH is similar, yet different, from hepatic AHH. Similar, in that AHH inducibility seems to be genetically regulated by the same locus, yet different, because there seems to be an organ specific response to MCA in even "noninducible" animals. This response may represent an adaptive response to the environment, since the lungs are constantly exposed to air and dust particles containing polycyclic aromatic hydrocarbons, insecticides, and other aromatic chemicals. This interpretation is in accord with the fact that skin is also slightly inducible in noninducible animals (WIEBEL et al., 1973).

These same strains of mice also respond by increased levels of pulmonary AHH to either whole cigarette smoke (Tables 4, 5, and 6) or particular fractions of condensates derived from this smoke (Table 7). Data are consistent with the observations of WELCH et al. (1971) and MARCOTTE and WITSCHI (1972), who showed that pulmonary AHH can be induced in rats exposed to regular or marijuana cigarettes. The lung seems to possess definite saturation levels for induction via cigarette smoke, for, regardless of schedule, only a 2 to 3 fold induction is observed. Exposure to 1, 2, or 3 cigarettes consecutively, or preexposure for up to 60 days with 8 cigarettes per day, yields quantitatively similar results. The AHH inducible C3H/fMai and B6 strains seem more responsive than the nonresponsive D2 strain (Table 5). However, after chronic exposure, both the C3H/fMai and D2 pulmonary tissues were induced (Table 6). Chronic exposure does not induce AHH levels in the liver, kidney, or intestines. Whether the pulmonary response of chronically smoked D2 mice represents true "induction" (e.g., utilizing the P-448 cytochrome) is presently being evaluated.

Results with the cigarette smoke condensate fractions (Table 7) demonstrate that the components of cigarette smoke that induce (or inhibit) pulmonary AHH can be discriminated. The relatively low inducing potential of whole cigarette smoke may reflect the presence of these inducing and inhibiting components. The BaP containing fraction, N_{NM}, is observed to be an effective inducer of pulmonary AHH and also an effective inhibitor of BaP metabolism *in vitro*. The chemical content of the potent $B_I a$ and $B_I b$ fractions is currently being determined. The phenol- or nicotine-containing fractions (WA_E and B_E) are observed to have little effect on AHH. The severe toxicity observed with the B_E fraction, however, may conceal any interaction between nicotine and AHH. Data in Table 8 nicely corroborate these IT results. Using a partially purified microsomal preparation of hepatic AHH, it was observed that certain fractions (e.g., $B_I a$, $B_I b$, and N_{NM}) are at least as inhibitory of BaP metabolism as 7,8-benzoflavone. Thus, there seems to be a correlation between ability to induce pulmonary AHH and ability to inhibit BaP metabolism *in vitro*. The most likely explanation is that these fractions contain compounds structurally similar to BaP; thus, they are capable of both inducing AHH and inhibiting BaP metabolism (competitively?) *in vitro*. The use of IT instillation of chemicals concomitant with tests for inhibition of BaP metabolism *in vitro* seems to produce rapid and reproducible tests for the detection of compounds that can potentially serve as inducers or substrates for the AHH system. Moreover, preliminary results from

the laboratory of AMES (U. of California, Berkeley) using these same smoke condensate fractions suggest that certain of these fractions (especially, $B_I a$, $B_I b$, WA_I, and neutral fractions) contain potent mutagenic activity (KIER et al., submitted, 1974). The relative carcinogenecity, and cocarcinogenecity, of these fractions are presently being tested both *in vivo* and *in vitro*.

Published results and preliminary results from our laboratory indicate pulmonary AHH may play a major role in lung cancer susceptibility. NETTESHEIM and HAMMONS (1971) reported conditions for induction of squamous cell carcinoma in inbred strains of mice. These authors utilized the (C57BL X C3H/f) F_1 and the DBA/2 strains of mice and 500 µg MCA (in 0.2% gelatin) given at weekly intervals for 4 to 6 weeks. The AHH inducible F_1 strain (KOURI, unpublished observation) was observed to be much more sensitive to MCA induced squamous cell carcinomas than the AHH "noninducible" DBA/2 strain. Very preliminary results from our laboratory involving IT administration of MCA into parent, F_1, backcross, and F2 animals (involving the B6 and D2 strains) indicate that AHH inducible mice seem to be more susceptible to MCA induced squamous cell carcinoma. Both results are compatible with the idea that the increased susceptibility to chemically induced carcinomas of AHH inducible animals reflects this heightened ability to metabolize chemical carcinogens.

F. Summary

The effects of exogenous factors on lung tissue of inbred strains of mice seem to be largely determined by the enzymatic activity of lung tissue itself. The major enzymatic activity studied in this paper was the inducible enzyme complex, AHH. It was shown that conditions could be developed so that pulmonary AHH levels were singularly effected. IT instillation of MCA (≤200 µg), exposure to whole cigarette smoke, and IT instillation of fractions of cigarette smoke condensate were shown preferentially to induce pulmonary AHH activity, and, in the case of MCA, this response was under host genetic control, segregating as a single autosomal dominant gene in crosses involving the B6 and D2 strains of mice. The small increase in D2 lung tissue following MCA treatment was attributed to nonspecific proliferation of enzymes similar to constitutive AHH, rather than a specific increase of the new P-448-mediated enzymes.

Exposure to whole cigarette smoke from either the 1A1 or 1R1 cigarette, using various exposure schedules, resulted in quantitatively similar increases in pulmonary AHH activity. Pretreatment for 60 days with 8 cigarettes per day did not increase this AHH response. Interposition of a Cambridge filter abrogated this enzyme response. Particular fractions derived from the smoke condensate of the 1A1 cigarette were observed preferentially to induce pulmonary AHH in B6 mice. These same fractions (e.g., $B_I a$, $B_I b$, N_{NM}) also were shown to inhibit BaP metabolism *in vitro*.

Results were discussed in view of the possibility that these enzymatic responses play a major role in the susceptibility of lung tissue to chemically induced cancers.

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References

BURKI, K., LIEBELT, A.G., BRESNICK, E.: Induction of aryl hydrocarbon hydroxylase in mouse tissues from a high and low cancer strain and their F_1 hybrids. J. nat. Cancer Inst. 50, 369-380 (1973).

CONNEY, A.H.: Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19, 307-366 (1967).

GILLETTE, J.R., DAVIS, D.C., SASAME, H.A.: Cytochrome P-450 and its role in drug metabolism. Ann. Rev. Pharmacol. 12, 57-84 (1972).

GOUJON, F.M., NEBERT, D.W., GIELEN, J.E.: Genetic expression of aryl hydrocarbon hydroxylase induction IV. Interaction of various compounds with different forms of cytochrome P-450 and the effect on benzo(a)pyrene metabolism *in vitro*. Mol. Pharmacol. 8, 667-680 (1972).

HO, W., FURST, A.: Intratracheal instillation method for mouse lungs. Oncology 27, 385-393 (1973).

KELLERMANN, G., CANTRELL, E., SHAW, C.: Variations in extent of aryl hydrocarbon hydroxylase induction in cultured human lymphocytes. Cancer Res. 33, 1654-1656 (1973a).

KELLERMANN, G., LUYTEN-KELLERMANN, M., SHAW, C.R.: Genetic variation in human lymphocytes. Amer. J. hum. Genet. 25, 327-331 (1973b).

KELLERMANN, G., SHAW, C.R., LUYTEN-KELLERMANN, M.: Aryl hydroxylase inducibility and bronchogenic carcinoma. New Engl. J. Med. 289, 934-936 (1973c).

KIER, L., YAMASAKI, E., AMES, B.: Detection of mutagenic activity in cigarette smoke condensates. (In press) 1974.

KOURI, R.E., SALERNO, R.A., WHITMIRE, C.E.: Relationship between aryl hydrocarbon hydroxylase inducibility and sensitivity to chemically induced subcutaneous sarcomas in various strains of mice. J. nat. Cancer Inst. 50, 363-368 (1973a).

KOURI, R.E., RATRIE, H., WHITMIRE, C.E.: Evidence of a genetic relationship between subcutaneous tumors and 3-methylcholanthrene-induced subcutaneous tumors and inducibility of aryl hydrocarbon hydroxylase. J. nat. Cancer Inst. 51, 197-200 (1973b).

KOURI, R.E., RATRIE, H., WHITMIRE, C.E.: Genetic control of susceptibility of 3-methylcholanthrene-induced subcutaneous sarcomas. Int. J. Cancer 13, 714-720 (1974a).

KOURI, R.E., RUDE, T.H., THOMAS, P.E., WHITMIRE, C.E.: Studies on pulmonary aryl hydrocarbon hydroxylase in inbred strains of mice. (In press) 1974b.

KUPFER, D., LEVIN, E.: Monooxygenase drug metabolizing activity in $CaCl_2$-aggregated hepatic microsomes from rat liver. Biochem. Biophys. Res. Commun. 47, 611-618 (1972).

MARCOTTE, J., WITSCHI, H.P.: Induction of pulmonary aryl hydroxylase by marijuana. Res. Commun. Chem. Path. Pharmacol. 4, 561-568 (1972).

MASON, H.S.: Mechanisms of oxygen metabolism. Adv. Enzymol. 19, 79-233 (1957).

NEBERT, D.W., GELBOIN, H.V.: The *in vivo* and *in vitro* induction of aryl hydrocarbon hydroxylase in mammalian cells of different species, tissues, strains and development and hormone states. Arch. Biochem. Biophys. 134, 76-89 (1969).

NEBERT, D.W., GOUJON, F., GIELEN, F.E.: Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons: simple autosomal dominant trait in the mouse. Nature New Biol. 236, 107-110 (1972).

NEBERT, D.W., GIELEN, J.E.: Genetic regulation of aryl hydrocarbon hydroxylase in the mouse. Fed. Proc. 31, 1315-1325 (1972).

NETTESHEIM, P., HAMMONS, A.S.: Induction of squamous cell carcinoma of the respiratory tract of mice. J. nat. Cancer Inst. 47, 697-701 (1971).

PATEL, A.R., HAQ, M.Z., INNERARITY, C.L., INNERARITY, L.T., WEISSGRABER, K.: Fractionation studies of smoke condensate samples from Kentucky reference cigarettes. Tobacco Sci. 1974 (in press).

ROBINSON, J.R., CONSIDINE, N., NEBERT, D.W.: Genetic expression of aryl hydrocarbon hydroxylase. Evidence for the involvement of other loci. J. Biol. Chem. 1974 (in press).

SLADEK, N.E., MANNERING, G.J.: Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Commun. 30, 607-612 (1966).

STOCKLEY, J.: Personal communication, 1974.

SWAIN, A.P., COOPER, J.E., STEDMAN, R.L.: Large scale fractionation of cigarette smoke condensate for chemical and biologic investigations. Cancer Res. 29, 579-583 (1969).

THOMAS, P.E., KOURI, R.E., HUTTON, J.J.: The genetics of aryl hydrocarbon hydroxylase induction in mice: A single gene difference between C57BL/6 and DBA/2J. Biochem. Genet. 6, 157-168 (1972).

WELCH, R.M., LOH, A., CONNEY, A.H.: Cigarette smoke: stimulatory effect on metabolism of 3,4-benzpyrene by enzymes in rat lung. Life Sci. 10, 215-221 (1971).

WIEBEL, F.J., LEUTZ, J.C., DIAMOND, L., GELBOIN, H.V.: Aryl hydrocarbon (benzo(a)pyrene) hydroxylase in microsomes from rat tissues: differential inhibition and stimulation by benzoflavones and organic solvents. Arch. Biochem. Biophys. 144, 78-86 (1971).

WIEBEL, F.J., LEUTZ, J.C., GELBOIN, H.V.: Aryl hydrocarbon (benzo(a) pyrene) hydroxylase: inducible in extrahepatic tissues of mouse strains not inducible in liver. Arch. Biochem. Biophys. 154, 191-194 (1973).

WHITMIRE, C.E., SALERNO, R.A., RABSTEIN, L.S., HUEBNER, R.J., TURNER, H.C.: RNA tumor-virus antigen expression in chemically induced tumors. Virus-genome-specified common antigens detected by complement fixation in mouse tumors induced by 3-methylcholanthrene. J. nat. Cancer Inst. 47, 1255-1265 (1971).

The Role of the Host in the Development of *in vivo* Models for Carcinogenesis Studies*

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ABSTRACT

The development of cancer depends upon the integrated response of the host to the carcinogen and to the initial transformation event. Genetic factors determine the host's potential to respond to chemical and viral carcinogens, while endogenous and exogenous environmental factors influence the realization of the genetic potential. In chemical carcinogenesis, inducibility (the capacity to metabolize polycyclic aromatic hydrocarbons (PAH) to the ultimate carcinogen) has been demonstrated to be under genetic control; however, tumors will occur to a lesser degree and after a longer delay when large doses of PAH are administered to noninducible mice. Aryl hydrocarbon hydroxylase induction by non-carcinogenic materials may also influence the effects of PAH carcinogens.

In viral carcinogenesis, evidence points to genetic transmission of the RNA oncogenic viruses with expression of the viral genome under host control. Host control over RNA tumor viruses may be demonstrated by the existence of epigenetic, xenotropic, and pantropic viruses; permissive, restrictive, producer, and nonproducer cell lines; and the presence of viral group specific antigen, infectious virus, and/or neoplasia in the host.

The development of cancer from the initial transformed cells (chemical and/or viral induced) is dependent on the host response which is influenced by numerous factors. Immunosurveillance is probably the first line of defense against these transformed cells. Genetic control of immunocompetence is evidenced by the variety of responses to various antigenetic stimuli in genotypically different strains of mice. Immunosuppressive effects of carcinogens, drugs, infections, etc., appear to make possible the initial act of establishing clones of transformed cells by overriding the immunocompetence of the host. Other factors related to diet, aging, stress, etc., effect the host control over the carcinogenic event and may be related to the increased susceptibility to carcinogens and/or the increase in the incidence of "spontaneous" tumors.

In our laboratory, we have undertaken studies to provide the best possible mouse model system for studying respiratory carcinogenesis. These studies with inbred mice have included the determination of relative susceptibility to various carcinogens, AHH inducibility, type C

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RNA viral expression, immunocompetence when challenged by various antigens, and the immunosuppressive effects of various chemical carcinogens. We plan to further evaluate our model systems with regard to environmental stress factors, cocarcinogenesis, and viral infections. In this way, we hope to establish the integrated host response to the events occurring in respiratory carcinogenesis.

Introduction

The ultimate solution of the cancer problem is dependent not only on the determination of the causes of cancer but also on the mechanisms whereby the host regulates the prevention and surveillance of the carcinogenic event(s) and the ultimate growth of the cancer. We must now ask what are the differences between susceptible and resistant hosts to the causes of cancer. Cancer, as a disease entity, differs in many respects from infectious diseases, in that there is a greater host-parasite involvement. Evidence points to cancer being an epigenetic disease (i.e., its development is dependent on an innate genetic resistance predisposed to gradual failure of control surveillance mechanisms). However, this is not the entire picture, since the response of the host to carcinogenic and subsequent events is an integrated response dependent on the interactions of both genetically controlled mechanisms and internal as well as external environmental factors.

A prerequisite to the etiological studies of any disease is the development of an appropriate animal model system. With the resolution of this problem, rapid progress in treatment and prevention can generally be made. Perhaps the greatest problem in carcinogenesis research has been the lack of a classical experimental animal model system. MEKLER (1973) has suggested that certain fundamental laws of biology pertinent to carcinogenesis have either escaped the attention of researchers or have not yet been discovered. The great strides which have been made in the study of host factors believed relevant to cancer have led us to the conclusion that we must better understand the role of the host in carcinogenesis before we can judiciously select the best animal model.

What species of laboratory animals provides us with the most information regarding those host surveillance mechanisms that may play a role in carcinogenesis? At this time, one would have to agree that there is more available information for various inbred strains of mice. For this reason, we suggest the mouse is still our best hope for selecting one or more model systems where inbreeding can provide the characterization of genetic factors related to carcinogenic susceptibility. There is probably no greater justification for using rats, rabbits, or for that matter, primates to demonstrate the carcinogenic potential of most chemicals. There are other justifications for the use of mice. Much of our knowledge regarding oncogenic viruses comes from mouse studies and has only recently been expanded to other species. In many ways, because of the broad knowledge of mouse genetics, we can come closer in mice to mimicking the situation that exists in man than in any other animal. Mice are easy to handle and require less space than most other animals; therefore, statistically significant results can be obtained by the use of large populations. Their relatively short life span of 2 to 3 years also makes them ideal subjects for lifetime studies. Their extraneous virus flora have been relatively well characterized and can be controlled by quarantine procedure. Their "spontaneous" tumor incidences have also been characterized.

There are of course various disadvantages, as is true with any model system. For respiratory carcinogenesis studies, mice have the disadvantage of being obligate nose breathers and this may present a different picture from that seen in man. Rats and mice have 4 nasal glands (vs 1 in man) which discharge fluids upon breathing noxious chemical or physical irritants. Mice also have more goblet cells per surface area of the respiratory epithelium (WYNDER and HOFFMANN, 1969). The organs and blood supply in mice are also small, thus presenting the disadvantage that only a limited number of studies can be undertaken with any one animal.

For the past 10 years, our laboratory has been actively defining certain parameters of viral-chemical carcinogenesis. Fortunately, other laboratories have pursued other aspects of genetically controlled surveillance mechanisms, which play important roles in cancer. It is our purpose in this paper to look at the integrated host (i.e., the inbred mouse) in an attempt to pull together many of the factors we feel play a role in the selection of the best possible animal model system for respiratory carcinogenesis. Our discussion of the mouse model system presents several aspects of host control over susceptibility:

- Susceptibility to chemical carcinogens
- Chemical carcinogen metabolism
- Viral etiology of cancer
- Tumor immunology
- Other genetic controls of neoplastic development
- Cells at risk to carcinogens and DNA repair

Chemical Carcinogenesis Susceptibility

During the past 7 years we have concentrated primarily on the characterization of various genotypically different mouse strains as to their susceptibility to 3-methylcholanthrene (MCA), 7,12-dimethylbenz(a)anthracene (DMBA), and benzo(a)pyrene (BaP) when given subcutaneously to female mice 4 weeks of age (WHITMIRE et al., 1971; WHITMIRE and SALERNO, 1972; KOURI et al., 1973b). These studies were based on an 8 month observation period, since most tumors occur during the first 5 to 6 months. The doses selected were relatively small, since our primary aim was to determine relative susceptibility in a reasonable period of time with as few adverse effects on the host as possible. The basis for comparison has been the cumulative tumor incidence, latency period (SHIMKIN and ANDERVONT, 1940), carcinogenic index (CI), (% cumulative tumors divided by the average days latency X 100) (IBALL, 1939), and tumor inducing dose in 50% of the animals in the defined observation period (TuD_{50}), (REED and MUENCH, 1938). We studied those mouse strains (inbred and random bred) most frequently used in cancer research. We have found correlation of the extensive literature difficult due to the numerous variables that inevitably exist between laboratories. Our results from studies carried out over an extended period using the same techniques, carcinogen dose, vehicle, and mice of comparable age and sex are presented in Table 1. Table 2 gives some of the host genetic factors for these inbred strains as a ready reference for later discussions.

These earlier studies with MCA have demonstrated a wide variation in the tumor incidence as well as average latency period among the genotypically different strains. Although the most susceptible strains generally develop tumors in the least amount of time, this is not

necessarily the case. For this reason the carcinogenic index (CI) has been utilized as a valuable index in rating the relative susceptibility of the various mouse strains (IBALL, 1939; WHITMIRE and SALERNO, 1972a). Probably the most outstanding examples of variation between tumor incidence and latency are the C3H/fMai and the C3H/HeJ, whose average latency is 14 weeks in each incidence but which produce 93% and 62% tumors respectively. Another example is that of the C3H/HeJ mice (14 weeks latency), which produce only 62% tumors, and the C57BR/cdJ strain, which produce 67% tumors with a relatively long average latency (22 weeks). These studies demonstrate that, although NIH Swiss, Ha/ICR, CFW, and CF-1 random bred strains have been used extensively in carcinogenesis studies, they are not as susceptible as a number of inbred strains. To define the relative susceptibility to MCA carcinogenesis still further, several dose levels were used and we found the C3H/fMai mouse strain to be consistently more susceptible than the other strains tested (Table 3). These studies also demonstrate a difference in mice of the same strain obtained from different sources. This is one variable in carcinogenesis studies of which most investigators are not aware and undoubtedly accounts for some of the variations in reported findings from one laboratory to another. The C57BL/6 mice from 3 sources act as entirely different strains (WHITMIRE and SALERNO, 1972a).

Table 1. Subcutaneous chemical carcinogenous characterization of various mouse strains with 150µg MCA

MOUSE STRAIN	TUMOR INCIDENCE Tu/T	%	AV. TU. LATENCY (WKS)	C.I.[a]	AHH[b] INDUCIBILITY	gs-1 ANTIGEN INCIDENCE P/T[c]	%	INFECTIOUS TYPE-C VIRUS
INBRED								
C3H/f Mai	51/55	93	13	102	9	16/23	70	±
C57BL/6 Cum	18/20	90	17	76	10	3/26	12	–
C58/J	20/23	87	16	76	10	10/10	100	+++
C57BL/10ScSnJ	22/27	81	18	64	12	0/14	0	–
BALB/cCr (Mai)	35/49	71	19	53	8	5/23	22	±
C57BR/cdJ	18/27	67	22	44	7	2/18	11	–
C3H/HeN	33/53	62	14	63	10	13/24	50	+
SWR/J	11/22	50	23	33	1	6/10	60	–
129/J	14/30	47	27	25	1	0/15	0	–
C57L/J	13/28	46	20	33	6	0/13	0	–
AKR/J	20/51	39	26	21	1	9/9	100	+++
SJL/J	13/35	37	22	24	1	11/15	73	++
DBA/2J	5/35	14	28	7	1	12/12	100	+
DBA/1J	6/53	11	27	6	1	4/8	50	+
RANDOM BRED								
SWISS-WEBSTER (N)	26/35	74	17	62	1	13/25	52	–
Ha/ICR (RPMI)	46/63	73	19	55	1	14/23	61	++
CFW	21/29	72	17	61	1	9/13	69	+
CF-1	37/66	56	23	33	1	15/15	100	++
SNELL/Mai	9/25	36	24	14	1	11/15	73	+

[a] CARCINOGENIC INDEX = $\frac{\%\ \text{TUMORS}}{\text{AV. DAYS LATENCY}} \times 100$

[b] AHH INDUCIBILITY = $\frac{\text{AHH LEVELS FROM MCA-TREATED MICE}}{\text{AHH LEVELS FROM TRIOCTANOIN TREATED MICE}}$

HEPATIC AHH LEVELS WERE DETERMINED 24 HOURS AFTER IP ADMINISTRATION OF 80 µg MCA/g BODY WEIGHT OR 0.05 ml TRIOCTANOIN. CONSTITUTIVE AHH LEVELS WERE SIMILAR FOR EVERY STRAIN TESTED

[c] $\frac{P}{T} = \frac{\text{NUMBER OF POSITIVE SAMPLES BY CF TEST}}{\text{TOTAL SAMPLES TESTED}}$

Table 2. Genes influencing AHH inducibility, tumor virus histocompatibility, and immunological responses

MOUSE STRAIN	GENE DESIGNATION[a]						IMMUNOLOGICAL RESPONSES[b] H-2 ASSOCIATED						NON H-2 ASSOCIATED			
							Ir-1 GENES SYNTHETIC POLYPEPTIDE COPOLYMERS (,)-A--L			GAT$_{10}$	Ir-Ig GENES BALB/c MYELOMA PROTEIN		Ir-3 GENES POLYPEPTIDES (,)-Pro-L		SC[c]	
	Ah	Fv-1	Fv-2	H-2	Ir-1	Ir-3	(T,G)	(H,G)	(Phe,G)		IgG	IgA	(H,G)	(T,G)	A	C
C3H/f	b	n	s	k	a	H9	L	H	H	H	L	H	H	H		
C57BL/6	b	b	r	b	b	H9	H	L	H	H	H	L	H	M		
C58	b	n	r	k	a	H9	L	H	H	H	L	H				
C57BL/10ScSn	b	b	r	b	b	H9	H	L	H	H	H	L				
BALB/c	b	b	s	d	a	H9	Mv	Mv	H	H	L	L				
C57BR/cd	b	n	r	k	a	H9	L	H	H	H	L	H				
C3H/He	b	n	s	k	a	H9	L	H	H	H	L	H	H	L		
SWR	d	n	s	q	c	H9	L	L	H		L	L	H	H	H	H
129	d	n	s	b	a	H9	H	L	H	H	H	L				
C57L	b	n	r	b	a	H9	H	L	H	H	H	L				
AKR	d	n	s	k	d	H-	L	H	H	H	L	H	H	L		
SJL	d	n	s	s	b	H9	L	L	L	neg	H	H	H	H	H	M
DBA/2	d	n	s	d	c	H9	Mv	Mv	H	H	L	L	H	L	H	M
DBA/1	d	n	s	q	c	H9	L	L	H		L	L	L	L	H	L

a STATTS, 1972

b McDEVITT AND LANDY, 1972

L = LOW
M = MEDIUM
Mv = MEDIUM VARIABLE
H = HIGH

[c]STREPTOCOCCAL CARBOHYDRATE ANTIGENS

Table 3. Comparison of tumor incidence, mean latency, CI and TuD_{50} in various strains of mice treated subcutaneously with various doses of MCA at 4 weeks of age

MOUSE STRAIN	9.38 µg TUMOR INCIDENCE Tu/T	%	MEAN CI LAT (WKS)		37.5 µg TUMOR INCIDENCE Tu/T	%	MEAN CI LAT (WKS)		150.0 µg TUMOR INCIDENCE Tu/T	%	MEAN CI LAT (WKS)		TuD_{50} µg MCA
AHH INDUCIBLE													
C3H/fMai	9/28	32	23	20	24/29	83	18	68	27/30	90	13	96	21
C3H/AnfCum	9/29	31	21	21	16/29	55	17	46	25/29	86	14	89	57
C57BL/6Cum	10/28	36	21	21	14/27	52	23	32	26/27	96	17	83	26
C57BL/6Mai	–	–	–	–	12/30	40	21	28	20/27	74	19	50	61
C57BL/6J	–	–	–	–	14/29	48	20	34	16/27	59	20	44	64
BALB/cCR (Mai)	3/30	10	27	7	17/28	61	20	45	23/28	82	16	71	34
BALB/cSPF (Mai)	–	–	–	–	20/30	67	17	57	26/31	84	12	98	≥38
C57BL/10ScSn	–	–	–	–	12/30	40	23	25	22/27	81	18	64	41
AHH NON-INDUCIBLE													
	150.0 µg				300.0 µg				500.0 µg				
129/J	14/30	47	27	25	16/28	57	21	39	20/28	71	22	46	203
DBA/2J	10/24	42	25	24	14/24	58	22	38	21/24	88	21	59	212
DBA/1J	4/27	15	25	9	13/24	62	25	35	10/19	53	23	33	238

The susceptibility of an animal to one carcinogen does not insure its responsiveness to another carcinogen. Therefore, studies were undertaken with DMBA and BaP in selected strains. As seen in Table 4, the C3H/fMai strain is highly susceptible to all 3 carcinogens, while the other strains are relatively insensitive to tumor induction with BaP and show varying sensitivity to DMBA. In all instances, the latency periods were longer with DMBA and BaP than with MCA. Although all polycyclic aromatic hydrocarbons are believed to be metabolized by the AHH enzyme system, there is evidence that their metabolism pathways are slightly different (NEBERT et al., 1973), which may account for genetic differences in the strains tested.

Chemical Carcinogen Metabolism

In order for polycyclic aromatic hydrocarbons (PAH) to exert cell transformation, mutagenicity, and tumor induction, they must be metabolized to water soluble active forms (e.g., the epoxide), (MARQUARDT and HEIDELBERGER, 1972; HUBERMAN et al., 1972) by the microsomal bound, mixed function oxidases of which aryl hydrocarbon hydroxylase (AHH) enzymes are a major system found in the tissues of man (KELLERMANN et al., 1973) and animals (GELBOIN, 1967). Constitutive enzyme levels are normally detectable without induction; however, the inducibility of AHH is associated with the carcinogenic effects of PAH (SELKIRK et al., 1971). The AHH system is inducible by a variety of endogenous chemicals (corticorsteroid hormones and bilirubin), as well as exogenous chemicals (barbiturates, insecticides, and PAH); therefore, these enzymes obviously may function as a two-edged sword. Our studies with various strains of mice have demonstrated a direct correlation between inducibility of AHH activity and 150µg MCA subcutaneous carcinogenesis (KOURI et al., 1973b). The results are summarized in Table 1. If, however, one gives higher doses of MCA (Table 3) to noninducible mice, the incidence of tumors can be increased, while the latency period remains at 5 to 6 months and the CI index below 60. The TuD_{50} dose is 3 to 11 times that of the inducible mice.

The role of AHH appears highly specific for each PAH. Thus, the metabolism of MCA, DMBA, and BaP does not necessarily follow the same pathways indicated by subcutaneous and skin carcinogenesis studies (KINOSHITA and GELBOIN, 1972; KOURI et al., 1973b; NEBERT et al., 1973). These differences in the carcinogenic effects (Table 4) indicate that the C3H/f mouse was the only strain capable of handling all 3 carcinogens equally well. It would appear that these variations in the AHH system could be clarified by studying congenetic crosses between the C3H/f strain and another strain giving low levels of tumor induction with BaP and DMBA and might help explain important etiological differences in chemical carcinogenesis.

The inducibility of the AHH system by various chemicals has been shown to be under host regulation. Susceptibility to MCA induction segregates as a single autosomal dominant gene in crosses involving the C57BL/6 (B6) and DBA/2 (D2) strains of mice (THOMAS et al., 1972; NEBERT et al., 1972b; GIELEN et al., 1972b). The B6 is the prototype inducible strain and its allele Ah^b designates the dominant gene. The D2 strain is the prototype strain for the recessive Ah^d allele. We utilized this mouse genetic system to extend our observations on the relationship between AHH inducible and sensitivity to MCA tumorigenesis (KOURI et al., 1973a, 1974c). The results in Table 5 demonstrate that inducible animals were approximately 10 times more sensitive to MCA carcino-

Table 4. Tumor incidence, mean latency, and CI in various strains of mice (females) inoculated subcutaneously with 150µg MCA, DMBA and BaP

	150 µg MCA				150 µg DMBA				150 µg BaP			
MOUSE STRAIN	Tu/T	%	MEAN LAT (WK)	CI	Tu/T	%	MEAN LAT (WK)	CI	Tu/T	%	MEAN LAT (WK)	CI
C3H/fMai	51/55	93	13.2	100	24/25*	96	17.5	78	20/24*	83	18.3	65
B10.BR/J	26/28	93	16.3	83	16/29	55	21.0	37	3/25	12	21.7	8
C57BL/6Cum	18/20	90	16.5	78	5/17	29	22.4	19	2/24	8	19.0	7
C57BL/10ScSn	22/27	81	18.1	64	10/24	42	22.8	26	4/29	14	24.0	8
129/J	14/30	47	27.0	25	11/33	33	26.5	18				
Snell/Mai	9/25	36	23.6	24	8/23	35	24.0	29	3/29	10	22.0	6

*THESE ANIMALS WERE MALES

genesis than noninducible animals when comparing the CI values. In every case where a tumor was observed on a noninducible animal, it occurred late in the observation period after tumor development had ceased in the inducible animals. It is assumed that some metabolism of this carcinogen took place at a much slower rate (possibly by the constitutive levels of AHH enzymes), leading to cell transformation and tumor induction.

The significance of AHH induction in the transformation of tissue culture cell lines by PAH carcinogens has been demonstrated (KOURI et al., 1974b). Only those cell lines potentially sensitive to chemically induced transformation possessed the particular type of metabolism involving the AHH inducible enzymes. The carcinogenic effect of a hydrocarbon is probably determined by the amounts activated to the carcinogenic form. The low levels of these enzymes in many tissue culture cell lines are probably a factor in the inability to obtain chemically induced transformation.

Enhancement and interference in the metabolism of chemicals by the AHH system can occur. CONNEY (1974) has demonstrated that oral treatment with MCA, DMBA, and BaP enhanced the metabolism of intravenously (IV) administered radioactive BaP in rats, while phenobarbital (which stimulates AHH induction) did not enhance MCA, BaP, or DMBA metabolism. Chronic administration of BaP stimulates the metabolism of radioaction BaP. These observations are of interest since treatment of rodents with AHH inducers provides protection for the carcinogenic effects of BaP, DMBA, N-2-fluorenylacetamide, 4-dimethylaminostibene, urethane, aflatoxin, diethylnitrosamine, and aminoazo dyes (CONNEY, 1974). THAMAVIT et al. (1974) have also reported that treatment with 3 carcinogens at one time decreases their carcinogenesis in rats and was believed due to an interference phenomena. WEBER et

Table 5. Relationship of hepatic inducibility and subcutaneous tumor induction with 150µg MCA in C57BL/6 x DBA/1, F1, and F2 mice

	AHH INDUCIBLE		AHH NON-INDUCIBLE	
	TUMOR INCIDENCE		TUMOR INCIDENCE	
MOUSE STRAINS	Tu/T	%	Tu/T	%
AHH NONSEGREGATING:				
B6 (Ah^b/Ah^b)	23/29	79		
D2 (Ah^d/Ah^d)			2/30	7
F1 (Ah^b/Ah^d)	54/90	60		
F1 x B6 (Ah^b/Ah^d and Ah^b/Ah^b)	81/94	86		
TOTALS:	158/213	74	2/30	7
AHH SEGREGATING:				
F1 x D2 (Ah^b/Ah^d and Ah^d/Ah^d)	15/24	75	5/34	15
F2 (Ah^b/Ah^b Ah^b/Ah^d Ah^d/Ah^d)	23/25	92	2/21	10
TOTALS:	38/45	84	7/55	13
TUMOR TOTALS:	196/258	77	8/85	11
AV. DAYS TUMOR LATENCY:	131		195	
CARCINOGENIC INDEX:-	59		6	

al. (1974) recently reported that nicotine reduced the metabolism of benzopyrene in tobacco smoke thus demonstrating interference between chemicals metabolized by the same enzyme system.

Not all known carcinogens belong to the group of chemicals known as PAH and their metabolism proceeds along different pathways. They have not been studied as extensively as the PAH group and the relationship to genetic patterns of susceptibility to carcinogenesis has not been determined. The dimethylase associated with the metabolism of the dimethylnitrosamines (DMN) may bear a mirror-image relationship to the AHH system, since high levels of induced AHH may act as potent repressors of DMN-dimethylase activity (VENKATESAN et al., 1971). This suggests that DMN may be a more potent carcinogen in AHH non-inducible animals. We are undertaking studies in AHH inducible and noninducible animals to test this hypothesis.

Virus Etiology of Cancer

Although a number of tumor viruses have been isolated from a variety of animal species since ROUS (1911) first discovered the avian sarcoma virus, the concept of viral etiology of cancer has been untenable for many scientists. The development of inbred mouse strains produced high and low incidence leukemia strains and ultimately led to the demonstration that RNA tumor viruses could be transmitted both horizontally and vertically (GROSS, 1944, 1950, 1970). Transmission can take place by congenital infection of the germ cells or via the placenta or milk; however, the usual mode of spread appears to be by genetic inheritance from one generation of animals or cells to the next as DNA copies of viral RNA integrated into the genetic material of the cells (WEISS, 1973; BUFFET et al., 1969; HILGERS et al., 1972).

Two concepts have been proposed for the origin of RNA tumor viruses: the oncogene hypothesis of HUEBNER and TODARO (1969) (TODARO and HUEBNER, 1972) and the protovirus hypothesis of TEMIN (1971, 1972). The oncogene theory suggests that viral genetic material is present in normal cells expressed as infectious virus or as noninfectious viral subunits (as viral group specific (gs) antigen) and that a noninfectious portion of the viral genome (the oncogene) is responsible for cellular transformation and cancer. The protovirus hypothesis differs from the oncogene hypothesis by suggesting that infectious viral genetic information is transferred by transcription and reverse transcription and, in combination with mutation or recombination events, produces neoplastic transformation.

Regardless of the hypothesis for the origin of the RNA tumor viruses, there are certain cellular controls which govern their expression. These controls will vary somewhat between endogenous and exogenous viruses; therefore, it is pertinent that some of the properties of each be considered. Endogenous viruses are transmitted vertically, either as viral genome or as infectious virus by congenital means. Multiple copies of the virogene are present in the DNA of all somatic and germ cells of all animals in a species. The type of viral expression is under cellular control and may be present as gs antigen, defective virus, or complete virus capable of growth under proper conditions with the production of reverse transcriptase (RT). Clonal lines established from these tissues will either spontaneously release virus or induce virus release after varying intervals of cultivation, depending on the original viral expression and the strength

or degree of cellular control. Induction can also be accomplished with 5'-iododeoxyuridine (IudR) or 5-bromodeoxyuridine (BudR). Complete virogene is known to be present in chickens, Chinese and Syrian hamsters, mice, rats, cats, pigs, and baboons and has been demonstrated by single cell clones with release of infectious virus. These cells with endogenous virogene are generally resistant to exogenous infection by the homologous endogenous virus. The expression of endogenous virus is influenced by the genetic properties of the virus and the cell as well as exogenous factors, such as radiation, chemical carcinogens, etc. Exogenous viruses differ from endogenous viruses in that they are spread horizontally as infectious virus (with evidence of RT) from animal to animal or cell to cell.

The characterization of the mouse type C viruses has recently been reviewed by SARMA and GAZDAR (1974). The type C RNA viruses from mice can be divided into 2 groups. The sarcoma viruses (MSV) produce solid tumors *in vivo* and transformed or cytopathogenic foci *in vitro*. These have been isolated rarely from laboratory adopted stocks of mouse leukemia viruses (MuLV), (HARVEY, 1964; MOLONEY, 1966; KIRSTEN and MAYER, 1971) or from several spontaneously occurring mouse sarcoma (FINKEL et al., 1966; GAZDAR et al., 1972). MSV can transform cells and release virus or they can fail to release virus, as seen in the nonproducer cell lines where the viral genome is integrated into the genetic material of the host cell. In such cases the viral genome can be rescued by superinfection with MuLV, which provides the envelope for the defective MSV. The mouse leukemia viruses (MuLV) are noncytopathogenic *in vitro* when propagated in permissive cells. *In vivo*, some produce leukemia while others fail to produce evidence of any neoplastic potential under the test conditions. They have been isolated from spontaneous and chemically induced solid tumors.

Recently, it has been shown that 2 classes of murine, RNA, type C viruses exist, based on their ability to replicate in mouse tissue. Those which will not replicate in mouse tissue but require rabbit, human, cat, etc., tissue are called xenotropic viruses (X-tropic), (LEVY, 1973) or S-tropic (SHERR et al., 1974). These viruses have typical murine type C antigenic markers but differ distinctly by nucleic acid hydridization from the N-tropic MuLV (BENVENISTE et al., 1974). The significance of the X-tropic viruses has not been determined at this time although they are apparently widespread. Their presence in the mouse with the apparent inability to propagate at least *in vitro* in mouse tissues presents an interesting type of genetic control that requires additional study. The implications of such viruses in humans provide a possible explanation to our inability to propagate a human cancer virus. MuLV, which replicate preferentially in mouse tissues, have been classified as ecotropic viruses (LEVY, 1974), and make up the group of murine viruses for which considerably more information is available. It is these viruses that will be discussed in this paper.

Vertical transmission of virogene information bypasses the host controls associated with infectious viral (but not necessarily oncogene) expression. Such host controls over infectious virus are common to vertically and horizontally transmitted infectious virus expression. Evidence of viral genetic information in the absence of infectious virus has been demonstrated by several systems. Virus-like molecule sequences were reported by HAREL et al. (1967) in the DNA of uninfected murine cells. CHASE and PIKO (1973) and VERNON et al. (1973) observed C-type particles, and gs antigen (HUEBNER et al., 1970a) has been demonstrated in embryonic tissues of mice. The $Gross_{IX}$ MuLV associated antigen was demonstrated by STOCKERT et al. (1971). Spon-

taneous and induced appearance of MuLV from clones of nonproducer cell lines has been demonstrated by TODARO (1972), AARONSON et al. (1969, 1971).

Based on our present technology for detecting type C RNA viral expression there appear to be 4 categories of mouse strains: 1. the C57L mouse expresses no viral antigen or infectious virus; 2. the NIH Swiss mouse has only the gs-1 antigen and no G_{IX} antigen, and the 129 strain expresses some gs-1 and G_{IX} antigen (neither of these 2 mice has infectious ecotropic MuLV, although type C particles have been observed in NIH Swiss mice); 3. certain mice have a low incidence of early ecotropic MuLV expression as gs-1 antigen that gradually increases and is accompanied by low levels of infectious MuLV expression as seen in the BALB/c mouse (PETERS et al., 1972a); and 4. a high level of infectious virus is detected early in life, accompanied by a high incidence of leukemia as demonstrated in AKR, C58, and C3H/Fi.

Several genes have been associated with endogenous virus expression; however, their roles have not been fully evaluated and may be related more to the expression of neoplasia than the virus per se. The TL antigen, determined by the Tla locus, may represent a viral genome since it appears only on leukemic cells and thymocytes of certain strains (BOYSE and OLD, 1969). The G_{IX} antigen is found on thymocytes and lymphocytes, but all murine leukemic cells are not G_{IX}^{+} (STOCKERT et al., 1971). TAYLOR et al. (1971) described 2 independent genes (no designation made) for gs antigen expression in the AKR mouse and postulated one locus for gs antigen expression and another for infectious virus in the AKR X C57BL/6 F2 cross. It is possible these 2 genes may be the same as the V_1 and V_2 loci for complete virus production in the AKR mouse described by ROWE (1972). These V-loci and the Ind locus (STEPHENSON and AARONSON, 1972a, 1972b) predispose cells to virus induction by IudR and BudR (ROWE et al., 1971). TAYLOR et al. (1973) also described the Mlv-1 allele in the C57BL/10 and DBA/2 strains as a determinate of gs antigen expression. Another gene (Fv-2) has been characterized for host control over propagation of the spleen focus-forming virus (SFFV) component of Friend virus complex. The susceptibility phenotype ($Fv\text{-}2^{s}$) is dominate and the $Fv\text{-}2^{r}$ denotes absolute resistance. This linkage is unrelated to the H-2 linkage and has no direct influence on the lymphatic leukemia virus of Friend (MCDEVITT and LANDY, 1972).

Interferon has been shown to be a potent antiviral agent and its production has been shown to be under host control (deMAEYER and deMAEYER-GINGNARD, 1969). This control is apparently related to the host response to various interferon inducers (BARON, personal communication, 1974) therefore demonstrating another variation in host control of infectious virus expression.

The best defined of the host cellular control genes for MuLV is that governing the replication of infectious virus. This spreading factor influences the ability of endogenous as well as exogenous ecotropic viruses to express themselves as infectious virus. The Fv-1 locus controls the host range permissiveness of mouse cells for the replication of MuLV (PINCUS et al., 1971a, 1971b). HARTLEY et al. (1970) demonstrated 3 groups of MuLV based on their ability to grow more efficiently in NIH Swiss (N) cells ($Fv\text{-}1^{n}$) or BALB/c (B) cells ($Fv\text{-}1^{b}$) and designated this predilection of the viruses as "N- and B-tropic" and NB-tropic" for those viruses that grow equally well in both types of cells. The tropism of the various mouse strains and their embryonic tissue culture cells have been classified as N-type or B-type. In the case of the MSV, the tropism is dependent on the tropism of the helper

virus. Mice strains that develop early leukemia belong to the Fv-1^n group (AKR, C58, C3H/Fi). These mice also carry the V_1 and V_2 alleles for high incidence activation of the endogenous MuLV. See Table 6 for summary of host genes associated with RNA type C viral expression control.

These type C viruses and the various host control mechanisms have been shown to play a role in the incidence of "spontaneous" (PETERS et al., 1972b) and virally induced neoplasia (LILLY and PINCUS, 1973; ROWE, 1972). They have been postulated to be switched on by chemical carcinogens (HUEBNER et al., 1970b, 1972; MEIER and MYERS, 1973); however, their significance in chemical carcinogenesis is still open to speculation. If one does undertake induced viral-chemical carcinogenesis studies, one must be cognizant of the susceptibility of the mouse strain not only to the chemical carcinogen used but also to the virus selected for these studies. It is also necessary to recognize the importance of endogenous viruses and the ensuing natural incidence of early or late development of leukemia when selecting inbred strains and congeneic strains for carcinogenesis studies related to the interaction of spontaneous and induced neoplasia (WHITMIRE et al., 1972b, 1972c, 1973a; SALERNO et al., 1973).

To determine the occurrence and concomitants of viral expression during MCA carcinogenesis in the various mouse strains, we have followed the incidence of gs antigen and infectious virus in the induced tumors (WHITMIRE et al., 1971, 1973b; WHITMIRE and SALERNO, 1972a). These results are summarized in Table 1. There is no significant correlation between susceptibility to MCA and the presence of viral expression as gs antigen or infectious virus. Other studies have confirmed this finding with DMBA and BaP (KOURI et al., 1973b). The gs antigen expression in chemically induced tumors follows the same pattern as that of the spleens of normal animals (MYERS et al., 1970) and could be related to the degree of expression that increases with age, as observed in the BALB/c mouse (WHITMIRE et al., 1973b).

Tumor Immunology

The significance of the host's ability to respond immunologically to the events of carcinogenesis was recognized early, yet today not all the ramifications of tumor immunology are understood. Further advances in technology and more knowledge regarding the integrated nature of host defense mechanisms might shed some light on this complex area. There exists the dichotomy between the healthy immune stimulus that provides for elimination of the initial transformed cells or the holding action in the host-parasite relationship, and the unhealthy condition where the immunological response enhances tumor growth. A point in cancer research has been reached when we must use the available knowledge regarding immunological competence for the selection of our animal models. It is my purpose to review some of these factors to be defined or at least recognized as existing in the animal models selected for the study of viral, chemical, and viral-chemical carcinogenesis. Sweeping conclusions regarding carcinogenic events can no longer be made in model systems without considering the integrated host reactions.

The metabolism of chemical carcinogens and factors related to viral etiology have already been considered; however, it will be necessary to consider the immunological response involved in those 2 facets that

Table 6. Genes influencing ecotropic mouse tumor virus expression (TOOZE, 1973)

ALLELE	PHENOTYPE	EXPRESSION	EXAMPLE STRAIN	REFERENCE
Tla	TL ANTIGEN	+	A, C58	BOYSE & OLD (1969)
		−	AKR, C57BL/6	
NOT DESIGNATED	gs ANTIGEN	+	AKR	TAYLOR, MEIER, MYERS (1971)
		−	C57L	
NOT DESIGNATED	INFECTIOUS MuLV	+	AKR	TAYLOR, MEIER, MYERS (1971)
		−	C57L	
V_1	N-TROPIC MuLV INDUCTION	V_1	AKR, C58, C3H/Fi	ROWE (1972)
		v_1		
V_2	N-TROPIC MuLV INDUCTION	V_2	AKR, C58, C3H/Fi	ROWE & HARTLEY (1972)
		v_2		
Ind	N-TROPIC MuLV INDUCTION	+	BALB/c	STEPHENSON & AARONSON (1972b)
		−	NIH-SWISS	
Fv-1	TISSUE TROPISM FOR VIRAL REPLICATION	n	AKR, C58	ROWE & HARTLEY (1972)
		b	C57BL/6, BALB/c	STEPHENSON & AARONSON (1972a)
		nb	NZB	
H-2	EARLY LEUKEMIA	k	AKR	BOYSE, OLD, STOCKERT (1972)
	LATE LEUKEMIA	b	AKR/H-2^b	
G_{1X}	G_{1X} ANTIGEN	+	129	BOYSE, OLD, STOCKERT (1972)
		−	C57L	
Mlv-1	gs ANTIGEN	a	C57BL/10, DBA/2	TAYLOR, MEIER, HUEBNER (1973)
		b		

allow the initial carcinogenic event to establish itself. Immunosuppressive effects of virus (SHEARER et al., 1973) and chemical carcinogens (STJERNSWÄRD, 1965; REES and SYMES, 1973; MATSUOKA et al., 1972; PARMIANI et al., 1971; BALL et al., 1966; BALL, 1970) undoubtedly play a significant part in the initial carcinogenic event. We are currently addressing ourselves to defining the immunosuppressive effects of various chemical carcinogens given intratracheally for the induction of lung cancer in several strains of mice (DEMOISE et al., 1974). Just as NETTESHEIM and HAMMONS (1971) have shown differences in susceptibility to lung carcinogenesis by MCA, STUTMAN (1969) indicates differences in the immunosuppressive effects in genotypically different strains. Based on our studies with MCA we would anticipate that both differences in tumor induction and immunosuppression may be correlated with the AHH inducibility of the various mouse strains (KOURI et al., 1973b).

The possible mechanisms whereby chemical carcinogens bring about carcinogenic events may be dependent not only on the transforming events but also on immunodepression, that allows these transformed cells to bypass the immunological defense mechanisms of the host. Immunosuppression by chemical carcinogens may, in fact, not be a single event, but cumulative, recurring events that allow frequent bypassing of host defense mechanisms and consequently slow interrupted but progressive growth of tumor cells.

The term "immunologic surveillance" coined by THOMAS (1959) and further expounded by BURNET (1970a, 1970b) denotes the idea of "seeking out and destroying" transformed cells by immunological means. This idea may not be totally correct, because rather than destruction, a "holding action" (LAPPE, 1971, 1972) may develop until such time as events in the integrated host's defense provide a favorable climate for the growth of the transformed cells (HESTON, 1963). This "sneaking through" event of some transformed cells that ultimately leads to the development of cancer is more of apparent scientific basis than it initially appeared. "Sneak through" has been thought to occur due to the location of the transformed cells in sites not exposed, or exposed less frequently to concomitant immunity. This unequal exposure to immune mechanisms is also believed to play a role in the site of metastites (VAAGE et al., 1971).

Another factor allowing "sneak through" of initial transformed cells is the low antigenic profile of some tumor cells as well as the low antigen load presented by only a few cells. For tumor cells to escape immune surveillance there must first be a barrier to escape. Non-immunogenic or low immunogenic tumors may not be capable of surveillance by immunological means. One must then define what we mean by "nonimmunogenic". Is it that these tumor cells are so like normal cells that they cannot be recognized as foreign by the host? Self-non-self discrimination is not fully understood and is intertwined with immunological tolerance, immunological paralysis, and immunological recognition. Is it that the host is tolerant to these cells since they contain certain embryonic antigens or endogenous viral genome antigens? Or, is it that the host is not capable of recognizing these antigens due to genetic variations within the species? This is where the inbred laboratory animals help us understand the role of genetic determinants in the immune process and, ultimately, to understand the events of neoplastic initiation and developments. Genetic variation in the immunological response can definitely influence neoplasia. Many of the differences in specific immunological responses have been linked to the histocompatibility gene in the mouse, rat, and guinea pig. Viral susceptibility has also been closely associated with the H-2 locus and such variations in susceptibility may be shown to be related, at least

in part, to inability to produce an immunological response in susceptible mice. These immune responses are under the control of individual dominant autosomal genes. Differences in response are not usually all or none but are concerned with quality and/or specificity of the antibody response.

The Ir-1 locus is closely linked to the H-2 gene in mice. Evidence indicates responsiveness to the various peptides (L-tyrosine and L-glutamic acid [T,G], L-phenylalanine and L-glutamic acid [P,G], and L-histidine and L-glutamic acid [H,G] built on multichain poly-DL-alanine) are under the control of different Ir-1 alleles (MCDEVITT, 1968). All of these polypeptides are antigenic in some strain of mice, but not necessarily in any one strain. They will not cross-immunize although antibody will cross react extensively. The Ir-1 gene functions at the T-cell level and controls cellular immune functions in the recognition of antigens, thus low responders are those who have reduced numbers of detectable precursor cells or cells with lower affinity for the specific immunogens. Low responder animals can, however, recognize these polypeptide antigenic determinants and produce large amounts of antibody when these hapten polypeptides are coupled with an immunogenic protein carrier. This shows one means of bypassing genetic defects (MCDEVITT, 1968).

The Ir-1 genes appear to have at least 2 or 3 separate loci. The Ir-IgA gene controls the immune response of mice to allotypic and idiotypic determinants on the IgA myeloma proteins derived from BALB/c mice. The Ir-IgG gene is linked to different H-2 specificities than the Ir-IgA and controls the immune response to γG (γ2a) of the same antigen (LIEBERMAN and HUMPHREY, 1971, 1972).

Various immunological responses have been shown to be non-H-2 linked. In immune responses to the (T,G) or (Phe-G)-Prol--L portion of multichain synthetic polypeptides, the response is controlled by the dominant, autosomal, Ir-3 gene (MOZES et al., 1973). The SJL mouse is the prototype for the high responders and the DBA/1 for the low responders. These responses are expressions of B cell activity and demonstrate antibodies can be made to 2 determinants on the same antigen under the control of 2 genes. Other non-H-2 linked gene controls of immune responses are reviewed by MCDEVITT and LANDY (1972), demonstrating dependence on the recognition of the antigenic determinant.

Antibody response to streptococcal polysaccharides differs dramatically with some strains, producing more to the group A than the group C carbohydrates, whereas in others, the reverse is true. These responses can give rise to a rather homogenous or a wide variation in the heterogeneity of the immune responses. B cell dependent responses to Salmonella lipopolysaccharides have also shown differences in mouse strain response and are dependent on recognition of the antigen (PAULI, 1972).

Having reviewed some of these various aspects of immunogenetic responses, one finds that new scientific discoveries are being made at a rate and volume beyond our ability to assimilate, evaluate, and make use of them as building blocks for rational progress. Unequal progress in the research prevents total integration of this knowledge into the understanding of host-parasite relationships. These studies with natural and synthetic antigens have demonstrated immunogenetic differences in responses which can not at this time be correlated with the viral and tumor specific membrane antigens. Recent advances in characterizing the amino acid sequences in the tumor virus (OROSZLAN et al., 1970; NOWINSKI et al., 1972) and the mouse myeloma protein (FRANCIS et al., 1974), the isolation of the viral envelope glycoproteins (KENNEL et

al., 1973), and studies with carcinoembryonic antigens (TOMITA et al., 1974) will lead to a greater understanding of their immunogenetic potential. Each host responds to some of these antigens but probably not to all of the exposed antigenic configurations making up the tumor cell membranes and soluble antigens. The immunological reactions summarized in Table 2 for the various mouse strains in our carcinogenic studies amplify the subtle difference in their responses to various defined immunogens. Those differences in the capabilities of the host to mount an adequate response to produce a "killer" or "holding" effect influence the capabilities of these genotypic strains to allow "sneaking through" events to occur for the establishment of the initial carcinogenic event as a pathologic entity and also to influence the process of metastasis and the blocking phenomenon (HELLSTRÖM and HELLSTRÖM, 1970) that accompanies rapid growth and terminal events of the neoplastic process. The genetic capabilities of the host to respond may be one of the important factors governing the variation in latency period of tumor development in the animal models as demonstrated in Table 1.

We have elected to characterize the susceptibility of a mouse strain not only by the tumor incidence and latency but also by the carcinogenic index (IBALL, 1939), which equates susceptibility to latency and incidence of tumor induction. It is obvious that certain strains have longer latency periods than others yet produce comparable numbers of tumors. We cannot say at this time whether this represents an inability to respond to certain tumor antigens allowing "sneak through" events to occur or whether these inbred strains actually are capable of mounting a high level of response leading to the blocking phenomena and insuring rapid tumor growth. HALPERN (1973) developed high and low responder lines of Swiss mice to various unrelated antigens. These 2 lines were clearly separated for their humoral responsiveness but showed similar cell mediated reactions. When allogenic Sarcoma 180 implants were made, the high responders allowed the tumor to grow and 90% of the animals were killed, while in the low responders, all tumors regressed. The high responders synthesized high levels of antibody believed to have allowed the tumors to grow due to the blocking reaction, while the low responders produced only enough antibody to provide for effective cellular immunity. If the type C viral antigens play a significant role in chemical carcinogenesis, it may well be that of providing antigenic components in the tumor cell surface which may make them more antigenic as postulated by BARBIERI et al. (1971) and GREENBERGER and AARONSON (1973). On the other hand, a tolerance to these antigens may exist or these endogenous viral antigens may act rapidly to overload the system leading to the blocking phenomena. It is difficult to speculate regarding the wide divergence in antigenicity of chemically induced tumors. It could be assumed, however, that either the individual mice have a wide variation in capability to respond or that the antigenicity of the various chemically induced tumors varies significantly accounting for variations in the immunological response. Such variations in capability to respond would influence the time required for the blocking type phenomena to develop, thereby influencing the rate of tumor development on an individual animal basis (BARTLETT, 1972). The latter of the 2 hypotheses seems most likely since we are dealing with inbred strains, although we have noted a wide variation between individual mice in the mixed lymphocyte studies. We need to define molecular serology of antigens, antibodies, and antigen-antibody complexes in order to study the mechanisms of immune interaction in the mouse model system where the science of immunogenetics is well advanced. Various sensitive serological procedures, such as the radioimmune assay, are available for analyzing the specific antigenic components of the tumor antigens.

Using such studies, we should be able to develop better diagnostic tools and, consequently, a knowledge of the potential usefulness of immunotherapy or immunological preventive procedures without producing an adverse stimulatofy effect to those clones of transformed cells maintained in a "holding" state by host control mechanisms.

Other Genetic Controls of Neoplastic Developments

Many known genes have been associated with the occurrence of various forms of neoplasms; however, in many instances this relationship appears to have no connection with cancer development (HESTON, 1972). Early experiments in cancer research were concerned mainly with the inheritance of susceptibility to spontaneous neoplasia. The development of the inbred strains and the use of congenic strains have allowed for more specific genetic analysis of host susceptibility to carcinogenesis. In most cases, it has been shown that carcinogenesis is dependent on multiple genetic and environmental factors. We will review only a few of the genetic linkages reported to influence cancer development.

HESTON (1963) reported linkage between pulmonary tumors and 8 specific genes (hr, A^y, vt, sh-2, wa-2, Fu, ah, and f), while LITTLE (1934), BITTNER (1945), and HESTON and DERINGER (1948) demonstrated linkage of mammary tumors with lethal yellow, brown, and agouti genes. STRONG (1945) linked gastric tumors with the brown gene while MACDOWELL (1945) and LAW (1952) demonstrated leukemia linked with dilute and flexed-tail genes. Although these associations have been made, their correlation with specific biochemical or physiological pathways has not been made. The H-2 histocompatibility loci has been associated with susceptibility or resistance to leukemia, the $H\text{-}2^k$ being considered susceptible while $H\text{-}2^b$ denotes resistance (LILLY, 1966). How the H-2 locus influences viral leukemia is not known but may represent influences discussed earlier in viral and immunological factor in the development of cancer.

MEIER et al. (1969) have described the genetic control of susceptibility or resistance to viral leukemogenesis by the hairless locus (hr). Hairless is an autosomal recessive mutation maintained in strain HRS/J. The incidence of leukemia is nearly 50% greater in the hairless (hr/hr) mouse than the haired mouse (hr/+) and occurs 6 months earlier. An N-tropic MuLV was isolated from both the hr/hr and hr/+ mice. The latency of neoplastic expression would appear to be related to an immunodeficiency factor rather than infectious virus expression (HEINIGER et al., 1974).

YAMAMOTO et al. (1973) propose that malignancy is induced by carcinogens by producing chromosomal changes resulting in a change in the balance between expression (E) and suppression (S) genes. E exists in normal cells but is neutralized by S, and malignancy occurs only when E increases or S decreases. Their studies with hamster cells injected with polyoma virus or treated with dimethylnitrosamine identified the location of the E and S chromosomes. These studies have been confirmed using Ara-C treatment of hamster cells (BENEDICT et al., 1974).

Cells at Risk to Carcinogens and DNA Repair Synthesis

In addition to the previously discussed genetic factors that provide varying degrees of protection against the initial carcinogenic event, there is an additional factor we wish to discuss: the cells at risk to carcinogenesis. Many factors influence this population of cells, as dose of carcinogens, chronic exposure, site of exposure, aging processes, hormonal influences, stress factors that induce hormonal changes, diet that influences protein metabolism, and promoters such as dust, asbestos, physical, microbiological and chemical irritants, etc. The list is long and cannot be fully exploited here. If, however, one examines this list closely, it is found these factors have one thing in common. They all influence some function of DNA repair. PIERCE (1970) points out that tumors arise only in those tissues capable of mitotic activity. Chemical carcinogens transform only those cells which are entering mitosis which makes up only 0.03% to 0.13% of the body's cells at any one time (MEKLER, 1973). The cells at risk are few and far between unless there are intervening factors that stimulate unscheduled DNA repair. STICH and SAN (1973) indicate a link exists between the oncogenicity of a compound and its capability to provoke DNA repair synthesis. This, however, is not the entire picture, since cocarcinogenic effects do occur requiring both inducers and promoters. Cancer research has made use of this process for years in the form of croton oil in back painting carcinogenesis experiments. This has been carried over into lung carcinogenesis with the use of ferric oxide with BaP (SAFFIOTTI et al., 1968, 1972) and carbon dust or aluminum oxide with BaP (HENRY and KAUFMAN, 1973). STANTON and BLACKWELL (1961) and BLENKINSOPP (1968) stimulated repair and regeneration of pulmonary epithelium by pulmonary infarction. Chronic respiratory infections also promote regeneration of lung tissue and may play a role in carcinogenesis.

The various factors involving DNA repair have not been fully defined. HEINIGER et al. (1972) determined the overall DNA-turnover in 19 inbred strains and F1 hybrid mice. The range of DNA turnover observed suggested polygenic control (H-1, H-3, and H-4) of the steady state. The mouse strains with the shorter turnover time were C57BR/cdJ, DBA/2, SWR/J, and BALB/c. The intermediate turnover rate was represented by C57BL/10, C57BL/6, AKR, C57L, SJL, C58, and C3H/He, while DBA/1 has the longest turnover rate, which was almost 3 times that of C57BR/cdJ. The DNA turnover rate showed no correlation with spontaneous tumorigenesis.

Significant differences in ulcer formation in mice has been observed (LILLY and DURAN-REYNALS, personal communication; NEBERT et al., 1972). The form of host control appears to be primarily that of cells at risk. Much of the body receiving the greatest assault from carcinogenic agents is extracorporeal and has special defense mechanisms. The skin is made up of stratified layers of epithelial cells, with those involved in mitosis being the least exposed to toxic substances. In the respiratory and alimentary systems there is a high degree of vascularization and lymphatic involvement and secretatory activity which tends to produce rapid detoxification and also provides a high level of immunological protection decreasing the incidence of carcinogenic "sneak-through" events occurring. The ciliary action of the respiratory tract also provides for elimination of particulate irritants that induce cellular division.

In the experimental animal models, most chemically induced tumors have had their origin in cells other than the epithilial cells. However,

in lung carcinogenesis, our primary concern is with the induction of squamous cell carcinomas. Alveoli are lined with squamous cells and are suspected to be the site of lung tumors. It is proposed that the use of ferric oxide or other particulate matter or chemicals that will induce mitotic activity increases the cells at risk and may in reality be only an exaggeration of what takes place in nature. Cigarette smoke carcinogenesis may be related to the cocarcinogenesis effects of weak carcinogens and particulate matter requiring extended chronic exposure. We have found that filtered cigarette smoke fails to induce AHH activity in the lungs of mice while unfiltered smoke induced for extended periods (KOURI et al., 1974). The particulate matter appears to play several roles, the trapping of AHH inducers and as promoters for weak carcinogen by increasing cellular proliferation. For these reasons it would appear the use of those agents that bring about unscheduled DNA synthesis are warranted in experimental models. Although we have considered primarily the influence of DNA synthesis on chemical carcinogenesis, this process is also important in triggering the hypothesized depression of repressors of tumor virus genome, protovirus, or oncogenes that may play an integrated role in the host as the etiological agents of cancer, be they viral, chemical or viral-chemical.

Summary and Application in Experimental Pulmonary Carcinogenesis Studies

The selection of animal models for cancer research must be based on the understanding and subsequent characterization of those host regulatory systems that define the differences between susceptible and resistant hosts. We have reviewed such host factors that appear to play decisive roles in carcinogenesis in the inbred mouse as an animal model. Differences in susceptibility to subcutaneous carcinogens with MCA, DMBA, and BaP have been demonstrated between various genotypically different strains of mice. Susceptibility to PAH carcinogens has been shown to be directly related to the inducibility of hepatic AHH, although differences in relative susceptibility to MCA, DMBA, and BaP were demonstrated in AHH inducible strains.

The host control of the type C RNA virus expression was reviewed. Although the frequency of occurrence of "spontaneous" neoplasia has been demonstrated to be related to infectious virus expression in mice, no direct influence of gs antigen or infectious virus expression on chemical carcinogenesis can be demonstrated. Various parameters of immunogenetics were discussed in relationship to the emergence of the initial transformed cells and the ultimate development of cancer. The immunosuppressive effects of carcinogens may play a role in the establishment of transformed cells and their growth. Differences in tumor latency are believed to be related to genetic differences in ability to recognize and respond to the various tumor cell antigens. The full impact of immunogenetics might be further understood when tumor cell antigens are more fully characterized.

We must consider not only the integrated host response but also the cells at risk to chemical carcinogens. Since DNA repair and mitotic activity may increase susceptibility to transformation, this aspect of susceptibility was discussed.

Based on our findings with subcutaneous chemical carcinogens in inbred mice, we have selected several strains for lung carcinogenesis studies. The effects of intratracheal inoculation of chemical carcinogens on AHH induction in the lungs (KOURI et al., these proceedings)

and host immunocompetence (DEMOISE et al., these proceedings) will be correlated with respiratory tumor induction. Studies are also in progress using ferric oxide in combination with chemical carcinogens in hopes of increasing the cells at risk and inducing higher incidences of squamous cell carcinomas. The use of wax pellet carcinogen implants (STANTON and BLACKWELL, 1961) is also being evaluated as a means of inducing lung cancers in mice. By examining these various parameters and methods of tumor induction, we will evaluate the inbred mouse as a model system for lung carcinogens.

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References

AARONSON, S.W., HARTLEY, J.W., TODARO, G.J.: Mouse leukemia virus: "Spontaneous" release by mouse embryo cells after long-term *in vitro* cultivation. Proc. nat. Acad. Sci. (Wash.) 64, 87-94 (1969).

AARONSON, S.A., TODARO, G.J., SCOLNICK, E.M.: Induction of murine C-type viruses from clonal lines of virus-free BALB/3T3 cells. Science 174, 157-159 (1971).

BALL, J.K.: Immunosuppression and carcinogenesis contrasting effects with 7,12-dimethylbenz(a)anthracene benz(a)pyrene and 3-methylcholanthrene. J. nat. Cancer Inst. 44, 1-10 (1970).

BALL, J.K., SINCLAIR, N.R., MCCARTER, J.A.: Prolonged immunosuppression and tumor induction by a chemical carcinogen injected at birth. Science 152, 650-651 (1966).

BARBIERI, D., BELEHRADEK, J., BARSKI, G.: Decrease in tumor-producing capacity of mouse cell lines following infection with mouse leukemia viruses. Int. J. Cancer 7, 364-371 (1971).

BARON, S.: Personal communication, 1974.

BARTLETT, G.L.: Effect of host immunity on the antigenic strength of primary tumors. J. nat. Cancer Inst. 49, 493-504 (1972).

BENEDICT, W.F., RUCKER, N., MARK, C., KOURI, R.E.: Correlation between the balance of specific chromosomes and the expression of malignancy in hamster cells. In preparation (1974).

BENVENISTE, R.E., LIEBER, M.M., TODARO, G.J.: A distinct class of inducible murine type C viruses which replicate in the rabbit SIRC cell line. Proc. nat. Acad. Sci. (Wash.) 71, 602-606 (1974).

BITTNER, J.J.: Inciting influences in the etiology of mammary cancer in mice. AAA Research Conference on Cancer, 63-96 (1945).

BLENKINSOPP, W.K.: Relationship of injury to chemical carcinogenesis in the lungs of rats. J. nat. Cancer Inst. 40, 651-661 (1968).

BOYSE, E.A., OLD, L.J.: Some aspects of normal and abnormal cell surface genetics. Ann. Rev. Genetics 3, 269-290 (1969).

BOYSE, E.A., OLD, L.J., STOCKERT, E.: The relation of linkage group IX to leukemogenesis in the mouse. In: RNA Viruses and Host Genome in Oncogenesis (P. Emmolot, P. Bentvelzen, eds), pp. 171-185. Amsterdam: North-Holland Publ. Co. 1972.

BUFFET, R.F., GRACE, G.T., DIBERANDINO, L.A., MIRAND, E.A.: Vertical transmission of murine leukemia virus. Cancer Res. 29, 588-595 (1969).
BURNET, F.M.: Immunological Surveillance. New York: Pergamon Press 1970a.
BURNET, F.M.: The concept of immunological surveillance. Prog. exp. Tumor Res. 13, 1-27 (1970b).
CHASE, D.G., PIKO, L.: Expression of A- and C-type particles in early mouse embryos. J. nat. Cancer Inst. 51, 1971-1975 (1973).
CONNEY, A.H.: Factors influencing benzo(a)pyrene metabolism in animals and man. In: World Symposium on Model Studies in Carcinogenesis (J. DiPaolo, P. Ts'O, eds). Cleveland: Chemical Rubber Co. (in press) 1974.
DEMAEYER, E., DEMAEYER-GUIGNARD, J.: Gene with quantitative effect on circulating interferon induced by Newcastle disease virus. J. Virol. 3, 506-512 (1969).
DEMOISE, C.F., KOURI, R.E., WHITMIRE, C.E.: Cell-mediated immunity after intratracheal exposure to 3-methylcholanthrene, and its relationship to tumor transplant growth in C3H/f Mai mice. In: These Proceedings.
FINKEL, M.P., BISKIS, B.O., JINKINS, P.B.: Virus induction of osteosarcomas in mice. Science 151, 698-701 (1966).
FRANCIS, S.H., GRALAM, R., LESLIE, Q., HOOD, L., EISEN, H.N.: Amino-acid sequence of the variable region of the heavy (alpha) chain of a mouse myeloma protein with anti-hapten activity. Proc. nat. Acad. Sci. (Wash.) 71, 1123-1127 (1974).
GAZDAR, A.F., CHOPRA, H.C., SARMA, P.S.: Properties of a murine sarcoma virus isolated from a tumor arising in an NZW/NZB F_1 hybrid mouse. I. Isolation and pathology of tumors induced in rodents. Int. J. Cancer 9, 219-233 (1972).
GELBOIN, H.V.: Carcinogens, enzyme induction, and gene action. Advanc. Cancer Res. 10, 1-81 (1967).
GIELEN, J.E., GOUJON, R.M., NEBERT, D.W.: Genetic regulation of aryl hydrocarbon hydroxylase induction. II. Simple mendelian expression in mouse tissues *in vivo*. J. biol. Chem. 247, 1125-1137 (1972).
GREENBERGER, J.S., AARONSON, S.A.: *In vivo* inoculation of RNA C-type viruses inducing regression of experimental solid tumors. J. nat. Cancer Inst. 15, 1935-1938 (1973).
GROSS, L.: Is cancer a communicable disease? Cancer Res. 4, 293-303 (1944).
GROSS, L.: "Spontaneous" leukemia developing in C3H mice following inoculation in infancy, with AK leukemic extracts or AK embryos. Proc. Soc. exp. Biol. (N.Y.) 76, 27-32 (1950).
GROSS, L.: Oncogenic Viruses. New York: Pergamon Press, Oxford 1970.
HALPERN, B.: Paradoxes in immunology. In: Mechanism in Allergy (L. Goodfriend, A.H. Sehon, R.P. Orange, eds), pp. 527-542. New York: Marcel Dekker, Inc. 1973.
HAREL, L., HAREL, J., HUPPERT, J.: Partial homology between RNA from Rauscher mouse leukemia virus and cellular DNA. Biochem. Biophys. Res. Commun. 28, 44-49 (1967).
HARTLEY, J.W., ROWE, W.P., HUEBNER, R.J.: Host range restrictions of murine leukemia viruses in mouse embryo cell cultures. J. Virol. 5, 221-225 (1970).
HARVEY, J.J.: An unidentified virus which causes the rapid production of tumors in mice. Nature (Lond.) 204, 1104-1105 (1964).
HEINIGER, H.J., CHEN, H.W., MEIER, H., TAYLOR, B., COMMERFORD, L.S.: Studies on the genetic control of cell proliferation: I. Clearance of DNA-bound radioactivity in 19 inbred strains and hybrid mice. Life Sci. 11, 87-96 (1972).
HEINIGER, H.J., MEIER, H., KALISS, N., CHERRY, M., CHEN, H.W., STONER, R.D.: Hereditary immunodeficiency and leukemogenesis in HRS/J mice. Cancer Res. 34, 201-211 (1974).

HELLSTRÖM, K.F., HELLSTRÖM, I.: Immunological enhancement as studied by cell culture technique. Ann. Rev. Microbiol. 24, 373-398 (1970).

HENRY, M.C., KAUFMAN, D.G.: Clearance of benzo(a)pyrene from hamster lungs after administration on coated particles. J. nat. Cancer Inst. 51, 1961-1964 (1973).

HESTON, W.E.: Genetics of neoplasia. In: Methodology in Mammalian Genetics (W.J. Burdette, ed.), pp. 247-268. San Francisco: Holden-Day, Inc. 1963.

HESTON, W.E.: Genetic Factors in Tumorigenesis. In: RNA Viruses and Host Genome in Oncogenesis (P. Emmolot, P. Bentvelzen, eds), pp. 13-24. Amsterdam: North-Holland Publ. Co. 1972

HESTON, W.E., DERINGER, M.K.: Relationship between the agouti gene and mammary tumor development in mice. Acta Univ. Internat. Centre de Cancer 6, 262-263 (1948).

HILGERS, J., BEYA, M., GEERING, G., BOYSE, E.A., OLD, J.J.: Evidence for mendelian inheritance. In: RNA Viruses and Host Genome in Oncogenesis (P. Emmolot, P. Bentvelzen, eds), pp. 187-192. Amsterdam: North-Holland Publ. Co. 1972.

HUBERMAN, E., KUROKI, T., MARQUARDT, H., SELKIRK, J.K., HEIDELBERGER, C., GROVER, P.L., SIMS, P.: Transformation of hamster embryo cells by epoxides and other derivatives of polycyclic hydrocarbons. Cancer Res. 32, 1291-1396 (1972).

HUEBNER, R.J., FREEMAN, A.E., WHITMIRE, C.E., PRICE, P.J., RHIM, J.S., KELLOFF, G.J., GILDEN, R.V., MEIER, H.: Endogenous and exogenous RNA tumor virus genomes in chemical carcinogenesis. In: Environment and Cancer, pp. 318-345. Baltimore, Maryland: The Williams and Wilkins Co. 1972.

HUEBNER, R.J., KELLOFF, G.J., SARMA, P.S., LANE, W.T., TURNER, H.C., GILDEN, R.V., OROSZLAN, S., MEIER, H., MYERS, D.D., PETERS, R.L.: Group-specific antigen expression during embryogenesis of the genome of the C-type RNA tumor virus: Implications for ontogenesis and oncogenesis. Proc. nat. Acad. Sci. (Wash.) 67, 366-376 (1970a).

HUEBNER, R.J., TODARO, G.J.: Oncogenes of RNA tumor viruses as determinants of cancer. Proc. nat. Acad. Sci. (Wash.) 64, 1087-1094 (1969)

HUEBNER, R.J., TODARO, G.J., SARMA, P., HARTLEY, J.W., FREEMAN, A.E., PETERS, R.L., WHITMIRE, C.E., MEIER, H., GILDEN, R.V.: "Switched off" vertically transmitted C-type RNA tumor viruses as determinants of spontaneous and induced cancer: A new hypothesis of viral carcinogenesis. In: International Symposium of the Centre National de Recherce Scientifique, Defectiveness, Rescue and Stimulation of Oncogenic Viruses, Vol. 183, pp. 33-57, Paris 1970b.

IBALL, J.: The relative potency of carcinogenic compounds. Amer. J. Cancer 35, 188-190 (1939).

KELLERMANN, G., LUYTEN-KELLERMANN, M., SHAW, C.R.: Metabolism of polycyclic aromatic hydrocarbons in cultured human leukocytes under genetic control. Humangenetik 20, 257-263 (1973).

KENNEL, S.J., DEL VILLANO, B.C., LEVY, R.L., LERNER, R.A.: Properties of an oncornavirus glycoprotein: Evidence for its presence on the surface of virions and infected cells. Virology 55, 464-475 (1973).

KINOSHITA, N., GELBOIN, H.V.: Aryl hydrocarbon hydroxylase and polycyclic hydrocarbon tumorigenesis: Effect of the enzyme inhibitor 7,8-benzoflavone on tumorigenesis and macro-molecule binding. Proc. nat. Acad. Sci. (Wash.) 69, 824-828 (1972).

KIRSTEN, W.H., MAYER, L.A.: Morphologic responses to a murine erythroblastosis virus. J. nat. Cancer Inst. 39, 311-335 (1971).

KOURI, R.E., DEMOISE, C.F., WHITMIRE, C.E.: The significance of the aryl hydrocarbon hydroxylase enzyme systems in the selection of model systems for respiratory carcinogenesis. These Proceedings.

KOURI, R.E., KIEFER, R., ZIMMERMAN, E.M.: Hydrocarbon metabolizing activity of various mammalian cells in culture. *In Vitro.* (In press) 1974b.

KOURI, R.E., RATRIE, H., WHITMIRE, C.E.: Evidence of a genetic relationship between susceptibility to 3-methylcholanthrene-induced subcutaneous tumors and inducibility of aryl hydrocarbon hydroxylase. J. nat. Cancer Inst. 51, 197-200 (1973a).

KOURI, R.E., RATRIE, H., WHITMIRE, C.E.: Genetic control of susceptibility to 3-methylcholanthrene-induced subcutaneous sarcomas. Int. J. Cancer 13, 714-720 (1974c).

KOURI, R.E., SALERNO, R.A., WHITMIRE, C.E.: Relationships between aryl hydrocarbon hydroxylase inducibility and sensitivity to chemically induced subcutaneous sarcomas in various strains of mice. J. nat. Cancer Inst. 50, 363-368 (1973b).

LAPPE, M.A.: Failure of long-term immunological control of 3-methylcholanthrene-induced skin tumors in the autochthonous host. J. Reticuloendothel. Soc. 10, 120-130 (1971).

LAPPE, M.A.: Possible significance of immune recognition of preneoplastic and neoplastic cell surfaces. In: Conference on Immunology of Carcinogenesis, DHEW Publication No. (NIH) 72-334. Nat. Cancer Inst. Monogr. 35, 49-55 (1972).

LAW, L.W.: The flexed-tail anemia gene (f) and induced leukemia in mice. J. nat. Cancer Inst. 12, 1119-1126 (1952).

LEVY, J.A.: Xenotropic viruses: Murine leukemia viruses associated with NIH Swiss, NZB and other mouse strains. Science 182, 1151-1153 (1973).

LEVY, J.A.: Autoimmunity and neoplasia: The possible role of C-type virus. Amer. J. clin. Pathol. (in press) 1974.

LIEBERMAN, R., HUMPHREY, W.: Association of H-2 types with genetic control of immune responsiveness to IgA allotypes in the mouse. Proc. nat. Acad. Sci. (Wash.) 68, 2510-2513 (1971).

LIEBERMAN, R., HUMPHREY, W.: Association of H-2 types with genetic control of immune responsiveness to I_GG (γ2a) allotypes in the mouse. J. exp. Med. 136, 1222-1230 (1972).

LILLY, F.: The histocompatibility 2 locus and susceptibility to tumor induction. J. nat. Cancer Inst. 22, 631-641 (1966).

LILLY, F., PINCUS, T.: Genetic control of murine viral leukemogenesis. In: Advances in Cancer Research (G. Klein, S. Weinhouse, A. Haddow, eds), Vol. 17, pp. 231-277. New York, London: Academic Press 1973.

LITTLE, C.C.: The relation of coat color to the spontaneous incidence of mammary tumors in mice. J. exp. Med. 59, 229-250 (1934).

MACDOWELL, E.C., POTTER, J.S., TAYLOR, M.J.: Mouse leukemia. XII. The role of genes in spontaneous cases. Cancer Res. 5, 65-83 (1945).

MARQUARDT, H., HEIDELBERGER, C.: Influence of "feeder cells" and inducers and inhibitors of microsomal enzymes on hydrocarbon induced malignant transformation of cells derived from mouse prostate. Cancer Res. 32, 721-752 (1972).

MATSOUKA, Y., SENOH, H., KAWAMOTO, T., KOHMO, T., HAMOAKA, T., KITAGAWA, M.: Failure of immunological memory in DMBA-treated mice. Nature New Biol. 238, 273-274 (1972).

MCDEVITT, H.O.: Genetic control of the antibody response. III. Qualitative and quantitative characterization of the antibody response to [(T,G)-A--L] in CBA and C57 mice. J. Immunol. 100, 482-492 (1968).

MCDEVITT, H.O., LANDY, M.: Genetic Control of Immune Responsiveness. New York, London: Academic Press 1972.

MEIER, H., MYERS, D.D.: Chemical co-carcinogenesis: differential action of various compounds, derepression of endogenous C-type RNA genomes, and influence of different genotypes of mice. In: Unifying Concept of Leukemia (Dutcher, ed.), Bibl. haemat., Vol. 39, pp. 551-573. Basel: Karger 1973.

MEIER, H., MYERS, D.D., HUEBNER, R.J.: Genetic control by the hr-locus of susceptibility and resistance to leukemia. Proc. nat. Acad. Sci. (Wash.) 63, 759-766 (1969).

MEKLER, L.B.: Chemical carcinogenesis. A new approach to the molecular and cellular mechanisms. Oncology 28, 68-82 (1973).

MOLONEY, J.B.: A virus induced rhabdomyosarcoma of mice. In: Conference on Murine Leukemia. Nat. Cancer Inst. Monograph. 22, 139-142 (1966).

MOZES, E., SHEARER, G.M., SELA, M., BRAUN, W.: Genetic control of immune responses to synthetic polypeptides in mice: Cellular analysis of the phenotypic correction of the Ir-3 gene defect by polyadenylic-polyuridiylic acid. J. Immunol. 111, 439-447 (1973).

MYERS, D.D., MEIER, H., HUEBNER, R.J.: Prevalence of murine leukemia virus group specific antigen in inbred strains on mice. Life Sci. 9, 1071-1080 (1970).

NEBERT, D.W., BENEDICT, W.F., BIELEN, J.E.: Aryl hydrocarbon hydroxylase, epoxide hydrase, and 7,12-dimethylbenz(a)anthracene-produced skin tumorigenesis in the mouse. Molecular Pharm. 8, 374-379 (1972a).

NEBERT, D.W., BENEDICT, W.F., KOURI, R.E.: Aromatic hydrocarbon-produced tumorigenesis and the genetic differences in aryl hydrocarbon hydroxylase induction. In: World Symposium on Model Studies in Chemical Carcinogenesis (J. DiPaolo, P. Ts'o, eds.). Cleveland: Chemical Rubber Co. (in press) 1973.

NEBERT, D.W., GOUJON, F.M., GIELEN, J.E.: Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons: Simple autosomal dominant trait in the mouse. Nature New Biol. 236, 107-110 (1972b).

NETTESHEIM, P., HAMMONS, A.S.: Induction of squamous cell carcinoma in the respiratory tract of mice. J. nat. Cancer Inst. 47, 697-701 (1971).

NOWINSKI, R.C., FLEISSNER, E., SARKER, N.H., AOKI, T.: Chromatographic separation and antigenic analysis of proteins of the onconaviruses. J. Virol. 9, 359-366 (1972).

OROSZLAN, S., FISHER, C.L., STANLEY, T.B., GILDEN, R.V.: Proteins of the murine C-type RNA tumor viruses. I. Isolation of a group-specific antigen by isoelectric focusing. J. gen. Virol. 8, 1-10 (1970).

PARMIANI, G., COLNAGHI, M.I., DELLA PORTA, G.: Immunodepression during urethan and N-nitrosomethylurea leukemogenesis in mice. Brit. J. Cancer 25, 354-365 (1971).

PAULI, R.D.: Genetics of the immune response. I. Differences in the specificity of antibodies to lipopolysaccharides among different strains of mice. J. Immunol. 109, 394-400 (1972).

PETERS, R.L., HARTLEY, J.W., SPAHN, G.J., RABSTEIN, L.S., WHITMIRE, C.E., TURNER, H.C., HUEBNER, R.J.: Prevalence of the group-specific (gs) antigen and infectious virus expressions of the murine C-type RNA viruses during the life span of BALB/cCr mice. Int. J. Cancer 10, 283-289 (1972a).

PETERS, R.L., RABSTEIN, L.S., SPAHN, G.J., MADISON, R.M., HUEBNER, R.J.: Indicence of spontaneous neoplasms in breeding and retired breeder BALB/cCr mice throughout the natural life span. Int. J. Cancer 10, 273-282 (1972b).

PIERCE, G.B.: Differentiation of normal and malignant cells. Fed. Proc. 29, 1248 (1970).

PINCUS, T., HARTLEY, J.W., ROWE, W.P.: A major genetic locus affecting resistance to infection with murine leukemia viruses. I. Tissue culture studies of naturally occurring viruses. J. exp. Med. 133, 1219-1233 (1971a).

PINCUS, T., ROWE, W.P., LILLY, F.: A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to Friend murine leukemia virus. J. exp. Med. 133, 1234-1241 (1971b).

REED, L.Z., MUENCH, H.A.: A simple method of estimating fifty percent endpoints. Amer. J. Hyg. 27, 493-497 (1938).

REES, J.A., SYMES, M.O.: Immunodepression as a factor during 3-methylcholanthrene carcinogenesis and subsequent tumour growth in mice. Int. J. Cancer 11, 202-211 (1973).

ROUS, P.: A sarcoma of the fowl transmissable by an agent separable from the tumor cells. J. exp. Med. 13, 397-411 (1911).

ROWE, W.P.: Studies of genetic transmission of murine leukemia virus by AKR mice. I. Crosses with Fv-1^n strains of mice. J. exp. Med. 136, 1272-1285 (1972).

ROWE, W.P., HARTLEY, J.W.: Studies of genetic transmission of murine leukemia virus by AKR mice. II. Crosses with Fv-1^b strains of mice. J. exp. Med. 136, 1286-1301 (1972).

ROWE, W.P., HARTLEY, J.W., LANDER, M.R., PUGH, W.E., TEICH, N.: Non-infectious AKR mouse embryo cell lines in which each cell has the capacity to be activated to produce infectious murine leukemia virus. Virology 46, 866-876 (1971).

SAFFIOTTI, U., CEFIS, F., KOLB, L.H.: A method for the experimental induction of bronchogenic carcinoma. Cancer Res. 28, 104-124 (1968).

SAFFIOTTI, U., MONTESANO, R., SELLAKUMAR, A.R., CEFIS, F., KAUFMAN, D.G.: Respiratory tract carcinogenesis in hamsters induced by different numbers of administrations of benzo(a)pyrene and ferric oxide. Cancer Res. 32, 1073-1081 (1972).

SALERNO, R.A., RAMM, G.M., WHITMIRE, C.E.: Chemical induction of subcutaneous tumors in BALB/c and Swiss mice infected with wild type-C RNA viruses derived from BALB/c tissues. Cancer Res. 33, 69-77 (1973).

SARMA, P., GAZDAR, A.F.: Recent progress in studies of mouse type-C viruses. Current Topics in Microbiology and Immunology (in press).

SELKIRK, J.K., HUBERMAN, E., HEIDELBERGER, C.: An epoxide is an intermediate in the microsomal metabolism of the chemical carcinogen, dibenz(a,h)anthracene. Biochem. Biophys. Res. Commun. 43, 1010-1016 (1971).

SHEARER, G.M., MOZES, E., HARAN-GHERA, N., BENTWICH, Z.: Cellular analysis of immunosuppression to synthetic polypeptide immunogens induced by a murine leukemia virus. J. Immunol. 110, 736-741 (1973).

SHERR, C.J., LIEBER, M.M., TODARO, G.J.: Mixed splenocyte cultures and graft versus host reactions selectively induce an "S-tropic" murine type C virus. Cell 1, 55-58 (1974).

SHIMKIN, M.B., ANDERVONT, H.B.: Comparative carcinogenicity of three carcinogenic hydrocarbons. J. nat. Cancer Inst. 1, 57-62 (1940).

STAATS, J.: Standardized nomenclature for inbred strains of mice: Fifth listing. Cancer Res. 32, 1609-1646 (1972).

STANTON, M.F., BLACKWELL, R.: Induction of epidermoid carcinoma in lungs of rats: A "new" method based upon deposition of methylcholanthrene in areas of pulmonary infarction. J. nat. Cancer Inst. 27, 375-407 (1961).

STEPHENSON, J.R., AARONSON, S.A.: Genetic factors influencing C-type RNA virus. J. exp. Med. 136, 175-184 (1972a).

STEPHENSON, J.R., AARONSON, S.A.: A genetic locus for inducibility of C-type virus in BALB/c cells. The effect of a non-linked regulatory gene on detection of virus after chemical activation. Proc. nat. Acad. Sci. (Wash.) 69, 2798-2801 (1972b).

STITCH, H.F., SAN, R.H.C.: DNA repair synthesis and survival of repair deficient human cells exposed to the K-region epoxide of benz(a)anthracene. Proc. Soc. exp. Biol. (N.Y.) 142, 155-158 (1973).

STJERNSWÄRD, J.: Immunodepressive effect of 3-methylcholanthrene. Antibody formation at the cellular level and reaction against weak antigenic homografts. J. nat. Cancer Inst. 35, 885-892 (1965).

STOCKERT, E., OLD, L.J., BOYSE, E.A.: The G_{IX} system. A cell surface alloantigen associated with murine leukemia virus, implications regarding chromosomal integration of the viral genome. J. exp. Med. 133, 1334-1335 (1971).

STRONG, L.C.: Genetic analysis of the introduction of tumors by methylcholanthrene. IX. Induced and spontaneous adenocarcinomas of the stomach in mice. J. nat. Cancer Inst. 5, 339-362 (1945).

STUTMAN, O.: Carcinogen-induced immune depression: absence in mice resistant to chemical oncogenes. Science 166, 620-621 (1969).

TAYLOR, B.A., MEIER, H., HUEBNER, R.J.: Genetic control of the group-specific antigen of murine leukemia virus. Nature New Biol. 241, 184-186 (1973).

TAYLOR, B.A., MEIER, H., MYERS, D.D.: Host gene control of C-type RNA tumor virus: Inheritance of the group-specific antigen of murine leukemia virus. Proc. nat. Acad. Sci. (Wash.) 68, 3190-3194 (1971).

TEMIN, H.M.: The protovirus hypothesis: Speculations on the significance of RNA-directed DNA synthesis for normal development and for carcinogenesis. J. nat. Cancer Inst. 46, 3-7 (1971).

TEMIN, H.M.: The protovirus hypothesis and cancer. In: RNA Viruses and Host Genome in Oncogenesis (P. Emmelot, P. Bentvelzen, eds), pp. 351-363. Amsterdam: North-Holland Publ. Co. 1972.

THAMAVIT, W., HAISA, Y., ITO, N., BHAMARAPRAVATI, N.: The inhibitory effects of α-benzene hexachloride on 3-methyl-4-dimethylaminoazobenzene and DL-ethionine carcinogenesis in rats. Cancer Res. 34, 337-340 (1974).

THOMAS, L.: Cellular and Humoral Aspects of Hypersensitive State (H.S. Lawrence, ed.), p. 530. New York: Hoeber-Harper 1959.

THOMAS, P.E., KOURI, R.E., HUTTON, J.J.: The genetics of aryl hydrocarbon hydroxylase induction in mice: A single gene difference between C57BL/6 and DBA/2J. Biochem. Genet. 6, 157-168.

TODARO, G.J.: "Spontaneous" release of type-C viruses from clonal lines of "spontaneously" transformed BALB/3T3 cells. Nature New Biol. 240, 157-160 (1972).

TODARO, G.J., HUEBNER, R.J.: The viral oncogene hypothesis: New evidence. Proc. nat. Acad. Sci. (Wash.) 69, 1009-1015 (1972).

TOMITA, J.T., SAFFORD, J.W., HIRATA, A.A.: Antibody response to different determinant on carcino-embryonic antigen (CEA). Immunol. 26, 291-298 (1974).

TOOZE, J.: The molecular biology of tumor viruses. New York: Cold Spring Harbor Laboratory 1973.

VAAGE, J., CHEN, K., MERRICK, S.: Effect of immune status on the development of artificially induced metastases in different anatomical locations. Cancer Res. 31, 496-500 (1971).

VENKATESAN, N., ARCOS, J.D., ARGUS, M.F.: Induction and repression of microsomal drug-metabolizing enzymes by polycyclic hydrocarbons and phenobarbital. Theoretical models. J. Theor. Biol. 33, 517-537 (1971).

VERNON, M.L., LANE, W.T., HUEBNER, R.J.: Prevalence of type-C particles in visceral tissues of embryonic and new-born mice. J. nat. Cancer Inst. 5, 1171-1175 (1973).

WEBER, R., COON, J.M., TRIOLO, A.J.: Nicotine inhibition of the metabolism of 3,4-benzopyrene, a carcinogen in tobacco smoke. Science 184, 1081-1083 (1974).

WEISS, R.A.: Ecological genetics of RNA tumor viruses and their hosts. In: Analytical and Experimental Epidemiology of Cancer T. Hirayama, ed.). Tokyo: Univ. of Tokyo Press 1973.

WHITMIRE, C.E., SALERNO, R.A.: RNA tumor virus gs antigen and tumor induction by various doses of 3-methylcholanthrene in various strains of mice treated as weanlings. Cancer Res. 32, 1129-1132 (1972a).

WHITMIRE, C.E., SALERNO, R.A.: Influence of preinfection of C57BL/6 mice with Graffi leukemia virus on 3-methylcholanthrene-induced subcutaneous sarcoma. Proc. Soc. exp. Biol. (N.Y.) 144, 674-679 (1973a).

WHITMIRE, C.E., SALERNO, R.A., MEROLD, V.M., RABSTEIN, L.S.: Effect of age at treatment and dose of 3-methylcholanthrene on development of leukemia and sarcoma in AKR mice. J. nat. Cancer Inst. 49, 1411-1415 (1972b).

WHITMIRE, C.E., SALERNO, R.A., RABSTEIN, L.S.: Effects of thymectomy, splenectomy and 3-methylcholanthrene on neoplasia expression, incidence and latency in AKR mice. Proc. Soc. exp. Biol. (N.Y.) 141, 890-894 (1972c).

WHITMIRE, C.E., SALERNO, R.A., RABSTEIN, L.S., HUEBNER, R.J.: RNA tumor virus antigen expression in chemically induced tumors. In: Unifying Concepts of Leukemia (R.M. Dutcher, L. Chiecobianchi, eds), Bibl. haemat., Vol. 39, pp. 574-588. Munich, Paris, New York: Karger 1973b.

WHITMIRE, C.E., SALERNO, R.A., RABSTEIN, L.S., HUEBNER, R.J., TURNER, H.C.: RNA tumor-virus antigen expression in chemically induced tumors. Virus-genome-specified common antigens detected by complement fixation in mouse tumors induced by 3-methylcholanthrene. J. nat. Cancer Inst. 47, 1255-1265 (1971).

WYNDER, E.L., HOFFMANN, D.: Bioassays in tobacco carcinogenesis. In: Progress in Experimental Tumor Research (F. Homburger, ed.), Vol. 11, pp. 164-193. Basel, New York: Karger 1969.

YAMAMOTO, T., RABINOWITZ, Z., SACHS, L.: Identification of the chromosomes that control malignancy. Nature New Biol. 243, 247-250 (1973).

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Cell-Mediated Immunity after Intratracheal Exposure to 3-Methylcholanthrene, and its Relationship to Tumor Transplant Growth in C3H/f Mai Mice

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ABSTRACT

Immunological deficiencies have often been observed to occur in association with cancer although the exact nature of this relationship has not been fully characterized. The relative immunocompetence of an individual definitely plays a major role in the ultimate susceptibility or resistance to cancer. Numerous studies support the concept that cell-mediated immunity (CMI) is largely responsible for the body's defense against cancer. Our laboratory is currently interested in the levels of chemicals at which tumorigenesis occurs in various strains of mice and whether immunocompetence of the animals is affected.

In this investigation C3H/f Mai mice were intratracheally instilled 4 times at weekly intervals with 500 µg of 3-methylcholanthrene dissolved in a corn oil vehicle. These treatments caused 8% lethality in 30 days; whereas vehicle alone is nontoxic. Effects on CMI were determined 3 days after each treatment by measuring rates of DNA synthesis with ^{3}H-thymidine in allogeneic and spleen lymphocyte cultures. Spleen, thymus, and lung weight as well as blood leukocyte counts were measured. Syngeneic and allogeneic tumor transplants were performed on control and test mice to determine whether CMI data is biologically relevant to the process of tumor growth. The CMI and tissue responses were again evaluated 7, 14, and 28 days after tumor transplantation.

Preliminary data indicates that CMI, as reflected in spleen lymphocyte responses to phytohemagglutinin, pokeweed mitogen and allogeneic antigen, was suppressed during intratracheal instillations of 3-methylcholanthrene. This effect was most pronounced in response to pokeweed mitogen and persisted at least 2 weeks after exposures were discontinued. Lymphocyte cultures from mice that received tumor transplants indicate that the earlier CMI inhibition produced by carcinogenic exposure is not only cancelled but actually enhanced although only syngeneic transplants were successful. Again, it will be of interest to follow the kinetics of this effect in the host and compare it to the rate of tumor transplant growth.

A. Introduction

The intratracheal instillation of polycyclic hydrocarbons in hamsters, mice, and rats has served as a useful model for studies of respiratory carcinogenesis (SAFFIOTTI et al., 1968; NETTESHEIM and HAMMONS, 1971; SCHREIBER et al., 1972; SAFFIOTTI, 1969). Our laboratory is currently interested in the physiological effects of some of these chemical

carcinogens, intratracheally instilled at concentrations known to induce respiratory malignancies. Evidence has accumulated during the past 15 years that clearly indicates the important role of host immunity in controlling the onset and progression of malignant diseases (MORTON, 1974). In this study as in others, we are characterizing some of the effects of chemical carcinogens on levels of host immunocompetence and their relationships to tumorigenesis.

B. Materials and Methods

I. Animals

Male C3H/f Mai mice 8 weeks old (Microbiological Associates, Walkersville, Maryland) and C57BL/6 Cum mice (Cumberland View Farms, Clinton, Tennessee) of similar age and sex were kept in disposable plastic cages containing corn cob bedding. They were given drinking water containing tetracycline (1 g/liter) and Purine Laboratory Chow ad libitum. A 12-hour lighting cycle was used.

II. Intratracheal Instillation of 3-Methylcholanthrene

C3H/f Mai mice were intratracheally instilled with 3-methylcholanthrene (MCA) according to the technique of HO and FURST (1973). Metofane (Pittman-Moore, New Jersey), an inhalation anesthetic, was used to anesthetize the mice. MCA (500 µg) was dissolved in 0.02 ml of corn oil (CO) and instilled in each of 100 test animals with a 19-gauge blunt needle attached to a Hamilton microliter syringe. A total of 4 such dosages were administered at 1 week intervals. Control mice (100) received only 0.02 ml CO.

III. Transplantation of Tumor Cells

Single-cell suspensions of syngeneic and allogeneic tumor cells from tissue cultures of a MCA induced C3H/f Mai tumor (passage 2) and a spontaneous BALB/c tumor (passage 2) (obtained from Dr. R. MADISON, Microbiological Associates) were diluted in Hanks' Balanced Salt Solution (1×10^7 cells/ml) for transplantation. Syngeneic and allogeneic tumor cells (1×10^6/0.1 ml) were inoculated into both MCA and CO exposed mice 2 days after the fourth, and final, intratracheal instillation. These were inoculated subcutaneously over the forehead for ease of palpation and measurement of growth.

IV. Culture Media, Mitogens, and Allogeneic Antigen

The culture media used was RPMI No. 1640 from Microbiological Associates supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 200 mM of L-glutamine. Phytohemagglutinin-M (PHA) and pokeweed (PW) mitogens purchased from Difco (Detroit, Michigan), and Gibco (Grand Island, New York), respectively, were reconstituted in sterile-distilled water and used at a final concentration of 1% v/v in culture media. Freshly prepared single-cell suspensions of spleen cells from C57BL/6 Cum mice were exposed to 4,000 R of X-irradiation and served as a source of allogeneic antigen.

V. Spleen Lymphocyte Culture

The relative reactivities of thymus-derived (T) and bone marrow-derived (B) spleen lymphocytes were distinguished *in vitro* by their responses to mitogenic challenge with PHA and PW respectively (ANDERSSON et al., 1972). T-cell activity was further assessed in the mixed lymphocyte culture (MLC) reaction in response to stimulation by allogeneic antigen (C57BL/6 Cum spleen cells) (PLATE and MCKENZIE, 1973).

Single-cell suspensions of spleen lymphocytes obtained from 5 individual mice sacrificed at regular intervals after each intratracheal instillation of MCA (3 days post-exposure) and at 7, 14, and 21 days after transplantation of tumor cells were prepared in the same manner for all *in vitro* assays of cell-mediated immune (CMI) activity. Spleens from MCA and CO mice were removed, their capsules opened with scissors, and the cells teased out into chilled media. The suspension was allowed to settle briefly to permit removal of connective tissue fragments and large cell clumps. After 3 centrifugations (1,000 rpm for 10 min) and rinses, the single-cell suspension of spleen lymphocytes was adjusted to a density of 6×10^6 cells per ml of media. For mitogen stimulated cultures, spleen lymphocytes (6×10^5/1.2 ml) were pipetted in quadruplicate aliquots into wells of Falcon No. 3040 plastic microtiter plates. PHA and PW were then added to give a final concentration of 1% v/v in each well. Media alone was used for unstimulated controls.

The assay for MLV activity was also set-up with 6×10^5 spleen lymphocytes per 0.2 ml of media per well from MCA or CO mice. Syngeneic (C3H/f Mai) and allogeneic (C57BL/6 Cum) X-irradiated spleen lymphocytes (6×10^5 cells in 0.2 ml media) were used for antigenic stimulation.

Both mitogenic stimulated and mixed lymphocyte cultures of spleen cells were incubated at 37°C for 48 hours in a 5% CO_2 atmosphere. One micro Curie of ^{3}H-thymidine was then added to each culture and incubated an additional 18 hours. After a total of 66 hours incubation, the cultures were harvested and the contents of each well were transferred to DEAE Whatman No. 81 filter pads and allowed to dry. These filters were washed 4 times with 5% dibasic sodium phosphate, followed by 5 washes with distilled water, and again dried. Filters were transferred to vials containing 10 ml of Liquifluor (Beckman Instruments, Fullerton, California) and counted in a Beckman model LS 250 scintillation counter. Responses of T and B populations of spleen lymphocytes were expressed as the difference in counts per minute (ΔCPM) between the unstimulated and mitogen stimulated cultures. Similarly, in the MLC reaction, the response of T cells was expressed as the difference in counts per minute (ΔCPM) between syngeneic and allogeneic antigen stimulated cultures.

VI. Host Tissue Measurements

Animal weights were obtained on all mice at weekly intervals and at times when mice were sacrificed for spleen lymphocyte cultures. Pooled thymuses and individual lung and spleens were weighed. The total number of peripheral blood leukocytes was obtained using a Fisher Autocytometer II (Fisher Instruments, Pittsburgh, Pennsylvania).

C. Results

I. Effects of MCA Exposure and Tumor Transplantation on Animal Weight

Animal weights of CO control and MCA test mice recorded after each intratracheal instillation and after transplantation of syngeneic and allogeneic tumor cells are shown in Fig. 1. After 4 exposures and 24 days on test, MCA-mice showed a 10% loss of weight compared to the CO controls. This effect persisted even after intratracheal instillations were discontinued, although by day 40 of the mice previously exposed to MCA did gain weight. It is interesting to observe that mice receiving syngeneic transplants (5 days after the last MCA treatment) unterwent a pronounced loss of weight through day 46, 20 days after tumors were transplanted. As presented later, syngeneic cells grew but allogeneic tumor cells did not.

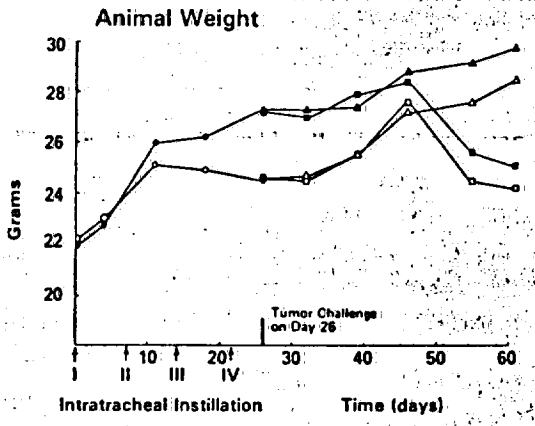


Fig. 1. Weight of mice in relation to intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

II. Effects of MCA on Host Tissue Response

Table I shows the effects of MCA exposure on thymus, lung, and spleen weights and on peripheral blood leukocyte counts. All of these tissues were noticeably altered. Loss of thymus weight ranged from -38% after 1 exposure to -60% after 4, whereas lung weight increased as a function of MCA exposure. Spleen weights were depressed during MCA instillation and rebounded after they were discontinued. Leukocyte counts appeared to have increased as a function of MCA instillation.

III. Growth of Tumor Transplants

Syngeneic and allogeneic tumor cells were transplanted in CO control and MCA test mice, but only the syngeneic transplants grew into tumors, as indicated in Table 2. Tumors were first detected by palpation in both CO and MCA mice at 10 days after transplantation. It is interesting that tumors in MCA-exposed mice were significantly ($p < 0.01$) smaller than those in CO mice at 13 days and were also somewhat smaller at 28 days after transplantation.

Table 1. Response of host tissues to 3-methylcholanthrene exposure[a]

Days on Test	No.[b] of Rx	ORGAN WEIGHT (grams) THYMUS C O	THYMUS MCA	THYMUS % Change	LUNG C O	LUNG MCA	LUNG % Change	SPLEEN C O	SPLEEN MCA	SPLEEN % Change	TOTAL CELLS (x 10^3) LEUKOCYTES C O	LEUKOCYTES MCA	LEUKOCYTES % Change
3	I	.047	.029	−38	.052	.069	+11	.065	.057	−12	3.2	2.9	− 9
10	II	.047	.028	−40	.228	.243	+ 7	.104	.088	−15	6.0	5.7	− 5
17	III	.055	.039	−29	.224	.250	+12	.068	.059	−13	5.0	6.0	+20
24	IV	.055	.022	−60	.214	.236	+10	.064	.061	− 5	4.0	7.0	+75
28	IV	.044	.027	−39	.186	.215	+16	.054	.070	+30	6.7	7.6	+13
35	IV	.033	.034	+ 3	.162	.191	+18	.064	.084	+31	6.6	8.0	+21

[a]Weights and cell totals represented as arithmetic mean of 10 mice

[b]Cumulative number of intratracheal instillations of 500 ug 3-Methylcholanthrene (MCA) in 0.02 ml corn oil (C O)

Table 2. Syngeneic and allogeneic tumor growth in 3-methylcholanthrene exposed mice

Tumor Type[a]	Treatment[b]	Tumor Size (cm) at Days After Transplantation 10	13	28	Tumored Mice No. Tested	(%)
Syngeneic	C O	palpable	0.94	1.91	26/26	(100)
	MCA	palpable	0.75	1.79	26/26	(100)
Allogeneic	C O	no tumor	0.00	0.00	0/26	(0)
	MCA	no tumor	0.00	0.00	0/26	(0)

[a]1 x 10^6 syngeneic (C3H/fMai) or allogeneic (BALB/c) tumor cells were injected subcutaneously in mice five days after the fourth and final intratracheal instillation

[b]Corn oil (C O) alone or 500 ug 3-Methylcholanthrene (MCA) dissolved in C O were administered intratracheally four times at weekly intervals before tumor transplantation

IV. Cell-Mediated Immune Effects of MCA Exposure and Tumor Transplantation

The relative reactivities of T and B spleen lymphocytes were determined *in vitro* with spleens removed from mice both during the intratracheal instillation of MCA and 7, 14, and 21 days after transplantation of tumor cells. Spleen lymphocyte responses to PHA, a T-cell specific mitogen, are shown in Fig. 2. Although some depression of T-cell activity occurred during intratracheal instillation of MCA, it reached significant levels only after the second ($p < 0.02$) and fourth (p 0.01) exposures. This depressed response returned to normal within 3 days after instillations of MCA were discontinued. One may observe that CO instillation in itself lowers T-cell activity after the second and third exposures. Most striking in Fig. 2 was the noticeable enhancement of T-cell reactivity produced by both syngeneic and allogeneic tumor cell transplantation regardless of whether the host was previously exposed to MCA or not. T-cell immune activity also was measured in response to allogeneic antigen in mixed lymphocyte cultures, and these results are shown in Fig. 3. Levels of T-cell reactivity were not effected by MCA exposure, but again were noticeably enhanced by transplantation of syngeneic and allogeneic tumor cells.

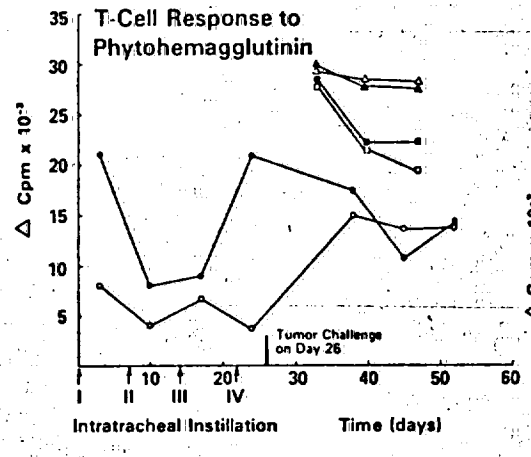


Fig. 2

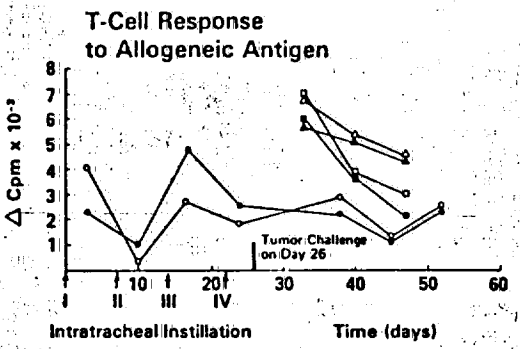


Fig. 3

Fig. 2. Response of spleen lymphocytes to mitogenic stimulation with phytohemagglutinin after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

Fig. 3. Response of spleen lymphocytes in mixed lymphocyte culture to allogeneic antigen after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

Whereas T-cell spleen lymphocyte activity was only periodically effected during MCA exposure, bone marrow-derived, B-lymphocyte activity was significantly depressed as shown in Fig. 4. Pronounced levels of depression occurred after instillations at 0 ($p < 0.0005$), 7 ($p < 0.01$), 14 ($p < 0.025$), and 21 ($p < 0.025$) days and remained depressed for 2 weeks after exposures were discontinued. Both T- and B-cell activity were stimulated by syngeneic and allogeneic transplanted tumor cells and again this occurred independent of whether the host had previously been exposed to MCA or not.

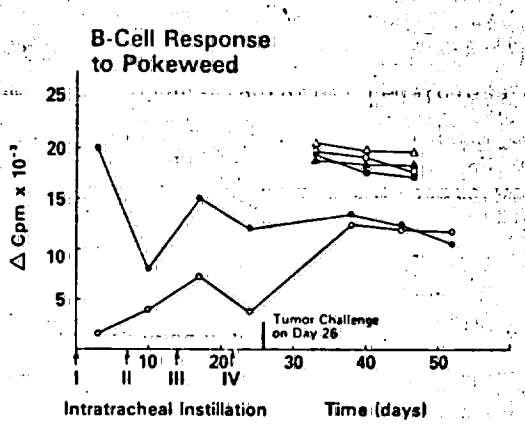


Fig. 4. Response of spleen lymphocytes to stimulation with pokeweed mitogen after intratracheal instillation of corn oil (solid circles) or 3-methylcholanthrene (open circles) and subsequent transplantation of syngeneic (squares) or allogeneic (triangles) tumor cells on day 26. Arrows indicate times of intratracheal instillation at 0, 7, 14, and 21 days

D. Discussion

Contrary to the expectation that MCA exposure might lower host immunocompetence to the point of allowing better growth of transplanted tumor cells, it instead exhibited a tumor-inhibitory effect, at least during the early stages of tumor growth. That transplanted syngeneic tumor cells did not grow as well in mice treated with MCA as in controls treated with CO might be related to the significantly depressed levels of B-cell activity incurred during the intratracheal instillation of MCA. This immunosuppressive effect might have been sufficient to influence at least the early stages of tumor growth. MCA is known to have an immunosuppressive property which impairs the function of B lymphocyte populations and thereby depresses the level of humoral antibody (BALL, 1970; STJERNSWÄRD, 1966; STUTMAN, 1969). Others have shown that antigens elicited on the surface of tumor cells can combine with circulating humoral antibodies to form antigen-antibody complexes effective in the prevention of tumor cell destruction by T-cell effector lymphocytes (BALDWIN et al., 1972, 1973; HELLSTRÖM et al., 1969). This being the case, lower humoral antibody levels in this study as indirectly suggested by the depressed B-cell activity in MCA exposed mice before tumor cell challenge would have permitted a more effective control of tumor growth. However, it is also possible that residual MCA in systemic circulation was simply cytotoxic to transplanted tumor cells and slightly inhibited their growth. Tumor inhibitory effects have been reported by others and have been compared to the deleterious action of x-ray, nitrogen mustard, methotrexate, and other agents on cell proliferation (HUGGINS and MCCARTHY, 1957; THOMPSON et al., 1960). It is difficult to separate the immunosuppressive and cytotoxic capacities

of various agents (STUTMAN, 1973). Of course, with regard to carcinogenicity, it has already been demonstrated that repeated intratracheal instillation of MCA in mice eventually produces respiratory malignancies (HO and FURST, 1973b; NETTESHEIM and HAMMONS, 1971). To whatever degree mouse immunocompetence was altered in this study, it was not sufficient to overcome a strong histocompatibility barrier and permit growth of transplanted allogeneic tumor cells.

Cell-mediated immune activity as demonstrated by B- and T-cell responses to mitogenic and allogeneic antigen stimulation was clearly enhanced by tumor transplantation regardless of any effect of MCA exposure or tumor growth. The 10% loss of weight by mice exposed to MCA might have been expected to produce a weakened physiological state and a specific loss of immunocompetence. Increased weight of lungs in exposed animals probably was caused by a collection of fluids and infiltration of lymphocytes as an inflamatory response to irritation. The pronounced loss of weight by thymus tissues in response to MCA might well have affected alterations in levels of CMI. Peripheral leukocyte counts were elevated in response to MCA and further reflected chronic irritation of the respiratory tract. However, cell-mediated immune activity was clearly stimulated in response to tumor transplantation and appeared to be independent of any previous MCA induced changes in thymus, lung, spleen, or leukocyte host tissues.

This study suggests that pulmonary exposure to polycyclic hydrocarbons in mice provides a useful model for characterization of the underlying mechanisms of respiratory carcinogenesis and host immunocompetence.

E. Acknowledgements

We gratefully acknowledge the expert technical assistance of SUSAN GOSNELL, CINDY MCKINNEY, CHARLES MURRAY, and KENNETH THOURSON. We thank Dr. ROBERT DONAHUE for a critical reading of the manuscript, and Mrs. VENTURA for its typing. This work was supported by The Council for Tobacco Research.

References

ANDERSSON, J., MOLLER, G., SJOBERG, O.: Selective induction of DNA synthesis in T and B lymphocytes. Cell. Immunol. 4, 381-393 (1972).

BALDWIN, R.W., EMBLETON, M.J., PRICE, M.R.: Inhibition of lymphocyte cytotoxicity for human colon carcinoma by treatment with solubilized tumor membrane fractions. Int. J. Cancer 12, 84-92 (1973).

BALDWIN, R.W., PRICE, M.R., ROBINS, R.A.: Blocking of lymphocyte-mediated cytotoxicity for rat hepatoma cells by tumor-specific antigen-antibody complexes. Nature New Biol. 238, 185-187 (1972).

BALL, J.K.: Immunosuppression and carcinogenesis: Contrasting effects with 7,12-dimethylbenzanthracene, benzo(a)pyrene, and 3-methylcholanthrene. J. nat. Cancer Inst. 44, 1-10 (1970).

HELLSTRÖM, I., HELLSTRÖM, K.E., EVANS, C.W., HEPPNER, G.H., PIERCE, G.E., YANG, J.P.: Serum mediated protection of neoplastic cells from inhibition by lymphocytes immune to their tumor specific antigens. Proc. nat. Acad. Sci. (Wash.) 62, 362-369 (1969).

HO, W., FURST, A.: Intratracheal instillation method for mouse lungs. Oncology 27, 385-393 (1973a).

HO, W., FURST, A.: Lung carcinogenesis by benzo(a)pyrene instillation. Proc. West. Pharmacol. Soc. 16, 146-149 (1973b).

HUGGINS, C., MCCARTHY, J.J.: Regression of human metastatic mammary cancer induced by 3-methylcholanthrene. Cancer Res. 17, 1028-1032 (1957).

MORTON, D.L.: Horizons in tumor immunology. Surgery 74, 69-79 (1974).

NETTESHEIM, P., HAMMONS, A.S.: Induction of squamous cell carcinoma in the respiratory tract of mice. J. nat. Cancer Inst. 47, 697-701 (1971).

PLATE, J.M.D., MCKENZIE, I.F.C.: "B"-cell stimulation of allogeneic T-cell proliferation in mixed lymphocyte cultures. Nature New Biol. 245, 247-249 (1973).

SAFFIOTTI, U.: Experimental respiratory tract carcinogenesis. Prog. exp. Tumor Res. 11, 302-333 (1969).

SAFFIOTTI, U., CEFIS, F., KOLB, L.H.: A method for the experimental induction of bronchogenic carcinoma. Cancer Res. 28, 104-124 (1968)

SCHREIBER, H., NETTESHEIM, P., MARTIN, D.H.: Rapid development of bronchioloalveolar squamous cell tumors in rats after intratracheal injection of 3-methylcholanthrene. J. nat. Cancer Inst. 49, 541-555 (1972).

STJERNSWÄRD, J.: Age-dependent tumor-host barrier and effect of carcinogen-induced immunodepression on rejection of isografted methylcholanthrene-induced sarcoma cells. J. nat. Cancer Inst. 37, 505-512 (1966).

STUTMAN, O.: Carcinogen-induced immune depression: Absence in mice resistant to chemical oncogenesis. Science 166, 620-621 (1969).

STUTMAN, O.: Immunological aspects of resistance to the oncogenic effect of 3-methylcholanthrene in mice. Israel J. Med. Sci. 9, 217-228 (1973).

THOMPSON, J.S., GURNEY, C.W., KIRSTEN, W.H.: The tumor-inhibitory effects of 3-methylcholanthrene-induced tumors in C3H mice. Cancer Res. 20, 1214-1219 (1960).

Experimental Lung Cancer

Carcinogenesis and Bioassays

International Symposium
Held at the Battelle Seattle Research Center
Seattle, WA, USA, June 23-26, 1974

Edited by
Eberhard Karbe and James F. Park

With 312 Figures and 144 Tables

Springer-Verlag Berlin Heidelberg New York 1974

Cigarette
Storage

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

March 4, 1975

To: The Scientific Advisory Board

Subject: Supportive Contract No. 20A - Microbiological Associates

The annual contract covers storage of cigarettes, purchased from the University of Kentucky, to be used in the inhalation and smoke fractionation studies of CTR contractors.

J.H.K.

RENEWAL

AGREEMENT BETWEEN

MICROBIOLOGICAL ASSOCIATES

AND

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

This agreement covers services (to wit storage of test cigarettes under controlled conditions) by Microbiological Associates (MA) for The Council for Tobacco Research - U.S.A. (CTR-U.S.A.) and dated December 12, 1974.

It is agreed that total charges for these services will not exceed twenty-five thousand dollars ($25,000) for the fifteen-month period beginning April 1, 1975 and running through June 30, 1976. These charges do not include shipping costs which will be billed separately to the CTR-U.S.A. Payment to MA will be made on a monthly basis in equal installments within twenty (20) days after receipt of an invoice.

The services to be performed by Microbiological Associates include the following:

Provide sufficient refrigerated space to store up to fifteen million (15,000,000) test cigarettes at a constant 0°-3°C temperature.

Receive additional subject test cigarettes on a schedule to be agreed upon mutually by the Project Director (MA) and the Project Officer (CTR-U.S.A.) as space permits in the present facility.

Insure that the security and accountability provided conforms with the extant regulations of the Alcohol, Tobacco and Fire Arms Tax Division of the U.S. Treasury Department.

Provide an appropriate alarm system and physical security to protect these cigarettes which are the property of The Council for Tobacco Research - U.S.A.

Provide adequate insurance coverage against loss by fire and other disaster.

Maintain a log-in and -out register of all receipts and issues. This record will be available for inspection by the Project Officer (CTR-U.S.A.) and/or the U.S. Treasury Tax Division on demand.

MICROBIOLOGICAL ASSOCIATES

BY: Vincent L. Ruwet

Vincent Ruwet
Vice President, Contracts and Administration

ACCEPTED:

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

_____

W. T. Hoyt
Executive Director

_____

Date

1003536801

Machine Evaluation
Dosimetry and Smoke
Fractionation

1003536803

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

March 4, 1975

To: The Scientific Advisory Board of The Council for Tobacco Research - U.S.A., Inc.

Subject: Oak Ridge National Laboratory (CTR Contract No. C15B). "Smoking Machine Evaluation, Animal Dosimetry and Smoke Fractionation Progress"

Oak Ridge, under CTR Contract No. 15 has been developing test methods and technology, analyzing and evaluating smoking machines for a number of parameters of central concern to the smoke inhalation program. As you are aware, the smoking machines have improved greatly in function and sophistication in the last two-year period, significantly as a result of Dr. Guerin's and Dr. Stokely's interest in this program and their ready collaboration with other investigators in and out of CTR.

Currently, as can be seen by the attached progress report and projected outline of the next year, they are investigating the chemistry and particulate characteristics of smoke produced in the Walton and P&I Sem I Prototypes. Some studies of smoke produced in the Lorillard LACS II are also anticipated once it is operational.

A large body of data has been accumulated, and they have begun to write a series of papers on relevant topics. These include vertical versus horizontal smoking, positive versus negative puffing; effects on smoke chemistry, particle size distribution in various machines using various assay measures, description of methods to characterize intermittent and continuous smoke generator machines, effects of interfaced animals and chamber constituent depletion, and several general papers on smoke chemistry and machine characteristics. Collaboratively several papers are possible in conjunction with Dr. Whitmire at MA, Dr. Essman at Queens College, Dr. Greenspan and Mr. Florant at P&I.

For the next year some additional assistance will be required to optimally develope the P&I Semi II model. It would not have been possible to design and build the Sem I in such a short span (since last January) without such a collaborative effort among CTR contractors. Extensive dosimetry studies, collaboratively with Drs. Whitmire, Kouri, Demoise at MA, and taking advantage of the analytical techniques developed in this program, are underway to define localized tissue content of smoke particulate and gas phase constituents. These studies will study different mouse strains on varied multiple smoke exposure regimens.

Lastly, a small pilot project to initate subfractionation of smoke condensate at no great cost would be begun. This program would not be extensive but should investigate feasibility of adopting technology currently applied to petroleum fractionation to our problems. It should

be clearly understood that this would be preliminary and not the extensive and more definitive colum chromotography, mass spectrographic program required to get answers rapidly in the future. Inasmuch as a preliminary program can give leads, it will be useful. Hopefully, some fractions could be tested for biological effects, such as IT and SC. cocarcinogenesis, AHH induction, S. typhimurium mutagenesis and specific tissue culture cell transformations.

J.H.K.

OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION
UNION CARBIDE
POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

February 14, 1975

Dr. John Kreisher
Associate Scientific Director
Council for Tobacco Research-USA, Inc.
110 East 59th Street
New York, New York 10022

Dear John:

I have attached my estimate of the accomplishments expected within the framework of existing third year funding and additional studies I believe worthy of supplementary funding as of 6-1-75.

Please let me know which, if any, of these additional studies should be included in a formal proposal for supplementary work/funding. I will prepare the formal request as soon as I hear from you.

You will note from Jim's Interim Progress Report that significant progress has been made. Our only complication has come in the major modifications and repair required of the LACS II and P&I SEM I.

You will be pleased to hear that considerable progress has also been made in preparing open literature publications. Considering deadlines, they will not appear in the literature before our more detailed Progress Report preparatory to third year funding is forwarded to you.

I look forward to hearing from you.

Sincerely,

M. R. Guerin

MRG
Attachment

APPENDIX I

PROJECTED CORE ACCOMPLISHMENTS*
(Without Supplementary Funding)

September 1975, 3 months into year 3

1. LACS II and P&I systems operational--at least well enough to test chemically.
2. Tests applied and results available as follows: mice present, ~10% smoke, 30/30 smoke/air cycle.

Measurement[1]	Purpose	Walton	P&I	LACS II
TC Monitor[2]	gp uniformity, age, purge	5 positions	7 positions	7 positions
Optical Monitor[2]	pm uniformity, age, purge	5 positions	7 positions	7 positions
GP Profile[3]	gp composition	1 position	1 position	1 position
TPM Profile[3]	pm composition	1 position	1 position	1 position
CO/CO_2	gp concentration, uptake/buildup	1 position	1 position	1 position
H_2O	humidity	1 position	1 position	1 position
Nicotine	pm concentration, nicotine uptake	1 position	1 position	1 position
Neophytadiene	pm concentration	1 position	1 position	1 position
Cost/Animal	cost/benefit	--	--	--
Mechanical	reliability, service requirements	--	--	--

(1) Relative to standard analytical smoke and nonrestrictive smoke.
(2) Continuous Measurement.
(3) Early and late (5 sec, 25 sec) in stand period.

December 1975, 6 months into year 3

1. Above, plus
2. Final report including following measurements: (a) DCBP or DTC dosimetry, (b) particle size growth and particle concentration.

June 1976, 12 months into year 3

1. Above, plus
2. Identification of constituents extracted preferentially from gas phase and particulate phase (determine whether change in composition is significant).
3. Particle size growth and concentration as functions of puff parameters, mode (normal, reverse; free, restricted) of puffing.
4. Complete comparative scheme, criteria, measurements.

APPENDIX II

"SUPPLEMENTARY" STUDIES

1. Special Measurements (BaP, HCN).

   a. BaP (Determination of trace constituent known to be carcinogenic). Method based on addition of carbon-14-labelled BaP to cigarette will be developed and applied to determine distribution of BaP in chamber(s). Determine whether measurements of nicotine, DCBP, DTC-C^{14}, and neophytadiene allow prediction of BaP concentration in unlabelled smoke and determine uptake by animals.

   b. HCN (Determination of known toxic and ciliatoxic constituent capable of reaction). Method based on standard colorimetric procedure or on nitrogen-selective gas chromatography will be developed and applied to determine concentration and distribution in chamber(s). Determine predictability of concentration from analytical measurements, loss to reaction if any, and uptake by animals.

2. Dosimetry by Depletion.

   It has been demonstrated (work for NCI, to be published) that the decrease in concentration of any constituent during the smoke stand period is a measure of the quantity of that constituent accepted by the animals, i.e., the dose of that constituent. Chamber depletion analysis thus allows determining the doses of actual smoke constituents and, being a non-destructive measurement, allows studying dose throughout a chronic exposure experiment.

   The proposed work:

   Using the mouse strain used in the Microbiological Associates chronic study, the depletions of nicotine, carbon monoxide, acetaldehyde, acrolein, isoprene, hydrogen cyanide (if 1-b is approved), catechol, glycerol, palmitic acid, phenol, and neophytadiene will be determined. Exposure conditions, including the test cigarette, will be the same as employed in the MICRO study.

   Above data will be studied to determine whether depletions of nicotine and carbon monoxide can be used to predict doses of other constituents and whether a constituent other than nicotine is a preferred particulate matter indicator for comparing exposure systems. Data will define MICRO exposure.

3. Multiple Inhalation Dosing

   Studies of the contribution of individual smoke constituents or smoke fractions to observed biological effects resulting from inhalation exposure require that techniques and instrumentation be available to allow multiple dosing. Studies of co-carcinogenic activity and individual susceptability also require or at least benefit from the availability of such instrumentation.

   Two approaches are possible. A pure compound, e.g., BaP, can be added to the cigarette to increase the concentration of that constituent in the smoke. Alternately, the compound or mixture can be metered into the chamber with the diluting air. The preferred approach depends on the experimental objectives--gases are best metered with the diluting air while some particulate components are best added to the cigarette.

The proposed work:

A carbon-14 distribution system of the type described by Philip Morris will be constructed and a carbon-14 sensitive gas chromatographic system will be purchased to allow studies of the fate of smoke constituents (selected for interest in co-factor exposure) added to the cigarettes. Constituents found transferred to the mainstream smoke without decomposition will be documented as applicable to this mode of co-exposure. Sufficient quantities of the constituent to be added will be synthesized or purchased and purified to prepare spiked cigarettes for inhalation testing.

The Walton-Horizontal system will be studied for the practicality of four co-factor dosing approaches: (a) pre-admission of freshly generated gas phase of smoke, (b) addition of gases and low molecular weight organics to fresh air supply, (c) pre-admission of gases and low molecular weight organics, and (d) pre-admission of particulates, smoke and non-smoke related, using an aerosol generator. Studies will include the development of suitable methods and hardware to produce the co-factor dose and chemically determining the concentrations and stability of the co-factor during the exposure period.

Co-factors to be studied using either cigarette addition and/or independent introduction will include carbon monoxide (stable gas), nitric oxide (reactive gas), acetaldehyde (low molecular weight organic), BaP (particulate carcinogen), smoke gas phase, classic tumor promoters, and an inorganic/organic aerosol yet to be selected.

4. Special Services for Microbiological Associates.

A "complete" chemical characterization of the smoke produced from the cigarette used for biological studies at MICRO would provide the following advantages: (a) direct comparability with NCI studies of approximately 120 cigarettes, and (b) "complete" definition of the exposure medium.

The proposed work:

Using both standard analytical smoke generation and standard but non-restricted analytical smoke generation, the following constituents will be determined in duplicate in the smoke of one cigarette type: TPM, water, nicotine alkaloids, tar, acetaldehyde, acrolein, isoprene, formaldehyde, oxides of nitrogen, hydrogen cyanide, carbon monoxide, carbon dioxide, whole smoke pH, catechol, phenol, free fatty acids, titrimetric acids, glycerol, and neophytadiene. Condensate (assumed produced by Meloy Labs under separate CTR contract) will be analyzed for indole, skatole, titrimetric acids, pH, BaA, BaP, phenol and cresols.

5. Subfractionation of Condensate by Chromatography--Pilot Study.

Future work for CTR-USA at this Laboratory will increasingly address quantitative fractionation of condensed smokes in support of biological studies by other CTR-USA contractors. A promising option to the sometimes criticized "Stedman-type" fractionation is the "SARA" chromatographic fractionation approach developed specifically for petroleum-related samples.

The proposed work:

The "SARA" chromatographic separation system will be constructed, applied to a petroleum-related sample to insure proper operation, and applied to condensed cigarette smoke. Aliquots of a given condensate will be subjected to the separation at least three times and the fractions obtained will be analyzed to determine quantitative and qualitative reproducibility. It will be known

whether the approach shows sufficient promise to warrent efforts to modify the systems for preparative scale operations.

APPENDIX II (page 3)

COST ESTIMATES

Supplementary Study	Man Year Ph.D	Man Year BS/MS	Man Year Tech.	Construction, Supplies, Equipment	Total Cost $/year	Completion Years
1. Special Measurements (BaP, HCN)	0.05	0.50	--	0.8 K	28.3 K	1.0
2. Dosimetry/Depletion	0.05	--	0.50	6.5 K	24.0 K	1.0
3. Multiple Dosing	1.00	1.00	--	25 K	125 K	2.0
4. Special Services, MICRO	0.05	--	0.50	0.5 K	18.0 K	1.0
5. SARA Fractionation	0.10	1.00	--	8.0 K	63 K	1.0
	1.25	2.50	1.00	40.8 K	258.3 K	

Cost Supplement	$258,300
Gas Chrom-Mass Spec	110,000
TOTAL COST	$368,300

100353812

OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION
UNION CARBIDE
POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

February 14, 1975

Dr. John Kreisher
Associate Scientific Director
Council for Tobacco Research-USA, Inc.
110 East 59th Street
New York, New York 10022

Dear John:

I have attached four copies of an Interim Progress Summary describing our work for the period June 1974 to January 1975. We have attempted in this summary to give an overview of the work rather than specific details. I will be happy to furnish additional information on projects that are of special interest.

Our work on the exposure machines is progressing along nicely. The new smoke distribution valve has been installed on the SEM I and is working well. The SEM I is being interfaced with our gas chromatographs to study smoke composition. We received the new model of the Walton from P&I recently and are doing preliminary work on it now. The LACS II is still giving us problems, but our maintenance people think most of the difficulties have been resolved, and the machine should be operational soon. One preliminary dosimetry experiment with Carrie has been completed and the data is in the computer now. John Caton and I will visit Carrie next week to make final plans and last minute changes in the experimental protocol. We are quite excited about the new method for particle size measurement and have applied it to the Walton. Our initial data on the Walton shows there is rapid growth of the particle size during the exposure period. It is going to be interesting to see what happens to the particle size with the P&I and Lorillard systems.

Mike and I are busy now writing papers. We hope to have drafts of three papers to you within three or four weeks.

We look forward to seeing you in the near future.

Sincerely,

James R. Stokely
Analytical Chemistry Division

JRS/bwm
Attachment

INTERIM PROGRESS SUMMARY

to

THE COUNCIL FOR TOBACCO RESEARCH--USA, INC.

on

CHARACTERIZATION OF ANIMAL EXPOSURE SYSTEMS

Prepared by

J. R. Stokely and M. R. Guerin
Analytical Chemistry Division
Oak Ridge National Laboratory

February 12, 1975

INTRODUCTION

This memorandum summarizes recent progress on the project to characterize animal exposure devices. Progress for the period June 1974 to January 1975 is summarized. Only abbreviated discussions of experimental results, conclusions, and the current status of the project are presented. Detailed discussions are not included as they are more appropriately presented in open literature publications and an annual progress report will be submitted in June 1975.

CURRENT STATUS

The primary objectives of the project are to establish a battery of tests for evaluating devices for exposing animals to whole tobacco smoke, to apply these tests to devices of interest to the Council for Tobacco Research, and to formulate recommendations for elimination of discovered shortcomings in the devices. Initial efforts were directed at developing methodologies needed to test the devices. This effort is continuing at a reduced level. Emphasis at the present time is on applying available tests to evaluate the Walton-Horizontal, Lorillard LACS II, and Process and Instruments SEM I exposure systems. A number of shortcomings in these systems have been discovered to date. Means for eliminating shortcomings have been devised and implemented in some cases.

Studies on the Walton Horizontal smoking machine have been extensive and have included an in-depth evaluation of the operational features of the machine and an investigation of the chemical properties of smoke offered to animals with the device. Chemical studies include assessment of smoke uniformity in the exposure chamber, losses of smoke from the exposure chamber, and the effect of smoke aging and animal interaction on the concentration and composition of smoke offered to animals with the device. Initial studies on smoke dose received by mice have been completed and more extensive studies are to begin in the near future. Methodology for determining the particle size distribution of smokes in animal exposure systems was recently developed. Initial application of the methodology has been made to the Walton system. Completed studies on the system are being prepared for open literature publication.

Work on the Lorillard LACS II exposure system has been restricted to an extensive operational evaluation and prelininary study of the chemical properties of smoke offered with the system. Complete evaluation of the system has been delayed because of the number and severity of maintenance problems that have been encountered. Although the basic design of the mechanical and electronic components of the system appear adequate, insufficient care was taken in construction of elec-

tronic circuits that control operation of the system. Continual failure of the electronic circuits has occurred, and a major effort has been required to locate and repair faulty circuits. The system is presently inoperative, and a number of electronic circuits are being reconstructed.

A prototype model of the Process & Instruments (P&I) SEM I exposure system was received during this report period and an extensive operational evaluation was made. Several shortcomings of the system were discovered and design changes were implemented. The design changes are being incorporated in a new model of the system which is presently under construction by P&I. Studies to define the chemical properties of smoke offered by the prototype system are currently underway.

ACTIVITIES FOR THE PERIOD JUNE 1974 TO JANUARY 1975

A. Operational Characterization and Design Improvements in the P&I SEM I System.

1. The electronic timing circuits which control operation of the smoke distribution valve did not permit accurate control of the exposure time. An improved timing circuit was devised and recommended to the manufacturer for inclusion in the final model of the system.

2. A temperature rise of from 65-70°C was found to occur in the vicinity of cigarettes during smoke generation. A temperature rise of this magnitude is not acceptable because of resulting changes in puff volume and because the chemical composition of the smoke may be altered. The temperature rise was reduced to 25-30°C by changing the design of the side-stream smoke hood and the air flow system. Further reduction in the temperature rise will require major design changes if chemical studies reveal that the smoke composition is affected.

3. The relative humidity of air in the dome area of the system decreases to less than 10% when several loads of cigarettes are smoked. Because of the possible effects of the low humidity on the burning rate of cigarettes, the chemical composition of the smoke, and deposition of smoke particulates in the animals, efforts are being made to humidify the dome supply air to 60% relative humidity at ambient temperatures. A device for injection of water into the air supply has been designed and built. The device is currently undergoing tests.

4. Fluctuations in dome pressure were encountered when the eject and loader assemblies operated. The fluctuations produced a change in the puff volume of from 3-5 ml. A ballast tank installed on the supply air system was found to reduce the pressure fluctuations to an insignificant level. The ballast tank will be incorporated into the final design of the system.

5. Operation and calibration of the SEM I is easy and uncomplicated. In about three months of heavy usage, no major mechanical breakdowns, component failures, or other maintenance problems have been encountered.

6. Measurements of the sidestream smoke concentration in the dome area were made using nicotine and carbon monoxide as smoke indicators. Based on these measurements, contamination of the mainstream smoke with sidestream smoke is less than 0.5%.

7. The static burn rate of cigarettes in the machine under operating conditions was measured. This study showed that the burn rate is not affected by the recommended air flow rate through the dome (350 cfm).

8. A number of minor design changes based on the initial evaluation of the prototype model were made to the manufacturer in a letter of January 2, 1975.

B. Chemical Properties of Smoke from the P&I SEM I System.

1. Assessment of the quantity and chemical composition of smoke generated and delivered by the prototype system is currently being made. Initial studies deal with the total particulate matter, nicotine, tar, and water deliveries of cigarettes smoked on the system. High resolution gas chromatographic methods for smoke particulates and gas phase are being applied to investigate smoke composition.

C. Operational Studies on the Walton-Horizontal Smoking Machine.

1. Measurement of the static burn rates of cigarettes showed that the machine would not smoke three cigarettes as designed. Air venting from the puffing dome flows over the cigarettes and causes the static burn rate to increase so that the cigarettes are consumed with less than the standard number of puffs. A solenoid valve was installed in the puff air supply to eliminate the problem.

2. The procedure for calibration of puff volumes with the machine has been simplified. Studies showed that the puff volume is not significantly affected by the resistance of the cigarette, eliminating the need for a separate calibration with each type cigarette smoked on the machine.

D. Chemical and Physical Properties of Smoke Presented to Animals with the Walton-Horizontal Smoking Machine.

1. The possibility of changes in smoke composition as the smoke stands in the exposure chamber was investigated using two high-resolution gas chromatographic profiling methods. These methods permit visualization of approximately 250 of the major organic components in the gas and particulate fractions of smoke. Profiles for smoke aged for 30 seconds were almost identical to fresh smoke indicating that very little or no compositional changes are occurring in the Walton exposure chamber during exposures up to 30 seconds.

2. Analyses were made for nicotine, nitric oxide, and neophytadiene in the Walton exposure chamber to determine the effect of aging on these important smoke components. Only with nitric oxide was aging shown to produce a change in the component concentration. With nitric oxide, the concentration decrease due to reaction with oxygen was very small--the concentration decreased less than 10% in a 30 second exposure.

3. The high resolution chromatographic profiling methods were used to establish the effect of animal interaction with smoke in the Walton exposure chamber. Appreciable interaction was found. Twenty mice cause a

general overall decrease in the gas and particulate phase concentration. In addition, several components in the gas phase are affected more than other components resulting in compositional changes. During a 30 second exposure, the overall organic gas phase concentration decreases by nearly a factor of two. One peak in the gas phase profile almost completely disappears and others are affected to a lesser extent. *Animal interaction with smoke in the Walton exposure chamber is potentially a serious problem* and should be taken into account in using the system for biological studies.

4. Analysis of nitric oxide in the Walton exposure chamber during exposures of 20 mice showed that the mice remove nitric oxide from the chamber at an appreciable rate and therefore deplete the chamber of this important smoke constituent. *The nitric oxide concentration is depleted by approximately a factor of two during a 30 second exposure of 20 mice.*

5. Application of newly developed methodology for particle size measurements have been made to determine the effect of smoke aging on the particle size of the smoke aerosol in the Walton exposure chamber. *Initial results show* that the particle size is increasing rapidly during a 30 second exposure; *the particle size approximately doubles during a 30 second exposure.* Studies on the effect of animals on the particle size are currently underway.

6. Comparisons were made of the composition of smoke generated by the Walton smoking machine and the Phipps and Bird analytical smoking machine. The high resolution chromatographic profiling methods were used for this comparison. This study showed that *smoke generated by the Walton duplicates smoke generated under standard smoking conditions.* They also suggest that *positive puffing has very little effect on smoke composition.*

7. *Continual use of the Walton machine* without cleaning *results in build-up of smoke particulates on the internal surfaces of the exposure chamber.* Studies were made to establish how frequently the exposure chamber should be cleaned to avoid exposing animals to the gas phase constituents in equilibrium with the particulates deposited on the walls of the chamber. Analysis of the gas phase from the exposure chamber for organic constituents showed very low levels of smoke components after 40 cigarettes had been smoked on the machine. *Once-a-day cleaning of the exposure chamber is sufficient* to prevent exposure to components in equilibrium with deposited particulates.

8. Initial dosimetry experiments with mice were performed during the first contract year on the Walton smoking machine. Extensive studies have been planned which will be a collaborative effort of this laboratory and Microbiological Associates. This work will begin with final approval from CTR.

E. Maintenance Problems with the Lorillard LACS II Exposure System.

1. Numerous maintenance problems have arisen in attempting to perform an evaluation of the Lorillard LACS II system. *The main cause for the problems is poor construction of electronic control circuits* in the instrument. During this report period, over 25 faulty solder joints have been repaired, and approximately 20 integrated circuits and 17 transistors have failed and have been replaced. The entire electronic circuits of the system are currently being checked and some circuits are being rebuilt. Thorough evaluation of the system is not possible until the electronic circuits are operating properly.

2. The quad-valve assembly on the system is inoperative. Lorillard Research has been informed of the mechanical failure of the valve, and is presently redesigning a new valve.

F. Preliminary Studies of Smoke Delivered by the Lorillard LACS II.

1. Measurements were made on the age of the smoke delivered by the LACS II using a thermal conductivity monitor developed during contract year one. These measurements showed that the smoke is less than 5 seconds old when it reaches the animal exposure unit (all of the smoke is less than 5 sec. old and 50% of the smoke is less than 2.5 seconds old).

2. Studies on the quantity of smoke delivered by the LACS II show that the smoke is offered to all animals at the same concentration. These studies were made using nicotine as a monitor of smoke concentration.

3. Losses of smoke particulates in connecting tubing used with the LACS II are very small and do not present a problem. Losses in the quad and shuttle valves have not yet been established.

4. The temperature in the vicinity of cigarettes smoked on the LACS II has been measured. It was found that the maximum temperature is 50°C which may have an effect on the chemical composition of smoke generated by the system.

G. Methodological Development and Exposure Concepts

1. A method has been developed for measurement of the particle size distribution of smoke particulates in exposure systems. The method involves encapsulation of smoke particles in a polymer bead and examination by scanning electron microscopy. Initial application of the method has been made to study smoke aging in the Walton Horizontal smoking machine.

2. Exploratory studies have been made on application of Fourier transform infrared spectroscopy to study compositional changes of smoke in animal exposure systems. Present indications are that the technique can be used to assess the possibility of very rapid chemical changes which occur within the first few seconds after smoke is generated. Techniques for detecting very rapid chemical changes are not now available.

3. A monitor for continuous measurement of the smoke particulate concentration in exposure systems is under development. Both laser light scattering and light absorption are being considered. A device is needed for particulate monitoring to establish smoke uniformity and concentration.

4. In smoke exposure systems, different smoke generation methods are used. Studies are underway to establish if and how the smoke generation method affects the delivery of the cigarette or the composition of the smoke. The following smoke generation variables are under study: horizontal vs vertical cigarette placement, restrictive vs free smoking, and positive vs negative puffing. Results obtained to date suggest that small but discernable differences in cigarette delivery result from the different smoke generation methods, but that compositional differences in the smoke are not evident.

The Council for Tobacco Research – U.S.A., Inc.

110 East 59th Street
New York, N.Y. 10022
(212) 421-8885

Contract
Application for Renewal of ~~Research~~ Grant
(#118)

1. Principal Investigator: Date: February 1, 1975

   Richard A. Lerner, M.D., Member

2. Institution and Address:

   Scripps Clinic and Research Foundation
   476 Prospect Street
   La Jolla, California 92037

3. Department where research will be done:

   Department of Immunopathology

4. Short Title of Study:

   Immunologic Competence and Chemical Carcinogenesis

5. Proposed renewal date:

   July 1, 1975

6. How results to date have changed earlier specific research aims:

   The specific research aim of this study continues to be the elucidation of the role played by the host immune mechanisms during chemical carcinogenesis.

   In Phase I of the study we selected the best assay of immunocompetence. We then utilized this assay to establish the immunocompetence of both sexes of six strains of mice to five different antigens.

   Phase II recently has been completed. It had previously been demonstrated that only strains which are so-called aryl hydrocarbon hydroxylase (AHH) inducible are susceptible to intratracheal (IT) chemical carcinogenesis by 3-methylcholanthrene (MCA). Essentially, we found that in these susceptible strains the IT administration of this carcinogen results in profound systemic immunosuppression. Phase III of the study will determine the importance of this immunosuppression in the development of lung tumors.

7. How results to date have changed earlier working hypothesis:

There has been no change in earlier working hypothesis.

8. Any additional facilities now required?

No.

9. Any changes in personnel?

No.

10. Append outline of experimental protocol for ensuing year.

Our results from Phase II of this research demonstrated that in certain strains of mice 500 µg MCA is immunosuppressive when given IT. This only occurs in strains of mice which are susceptible to induction of lung tumors by IT MCA.

The next series of experiments will attempt to dissect chemical carcinogenesis from immunosuppression in this system. This is important because the approach to the control of lung tumor induction would differ considerably depending upon whether the immunosuppression we observed was the permissive factor permitting tumor growth, or was merely a biologic event having no etiologic import.

Since C_3H strain mice are sensitive to induction of lung tumors with MCA administered IT and also exhibit significant immunosuppression, we will utilize mice of this strain exclusively. The first experiment will be directed towards finding a dose of carcinogen that is sufficient to induce lung tumors but does not cause immunosuppression.

Mice will be given intratracheally either saline, gelatin, 9.38 µg, 18.75 µg, 75 µg or 300 µg of MCA at Microbiological Associates, Inc. (MAI). Mice are coded as to dosage of carcinogen or control by marking specific toes, and the assays are performed at Scripps without knowledge of dosage of carcinogen administered (i.e., blind). They will then be shipped from MAI to Scripps Clinic and Research Foundation and six days after intratracheal inoculation, mice will be immunized with either goat erythrocytes or saline. Ten days later, the mice will be re-immunized, and either three, five, or seven days thereafter, the number of cells in each spleen secreting antibodies to goat erythrocytes will be assayed by utilizing the Jerne plaque assay. This will be performed on a random integrated schedule so that control and experimental animals are carefully co-mingled each day. The calendar for this study is attached. Each manipulation noted on the calendar involves fifty individual mice.

This study will be completed by mid-year. The code will be broken and the data analyzed. We will then select a low dose of MCA, below the immunosuppressive threshold, and examine the immune response after multiple exposures to this low dosage.

Longer range plans are directed towards defining the role played by immunosuppression in permitting tumor development after chronic low dose exposure to chemical carcinogens and/or smoke.

11. List publications or papers in press resulting from this or closely related work.

Manuscript in preparation.

12. Summary progress report:

During the past year the second phase of this study was completed. This study was designed to determine whether or not doses of MCA sufficient to cause lung tumors when given IT also caused immunosuppression. Briefly, we found that in strains of mice that are sensitive to MCA carcinogenesis, systemic immunosuppression is observed after MCA is administered. Resistant strains of mice fail to exhibit systemic immunosuppression after MCA administration.

It has previously been shown that production of the aryl hydrocarbon hydroxylase (AHH) family of enzymes is induced by MCA, as well as other polycyclic aromatic hydrocarbons (PAH) in some strains of mice but not in others. The PAH are the substrate of these enzymes. MCA is only carcinogenic in the strains of mice in which it induces the AHH enzymes, and it is also only immunosuppressive in these strains of mice. Strains of mice that cannot catabolize MCA are not immunosuppressed by it, and do not develop tumors. This very important finding links immune competence to chemical carcinogenesis.

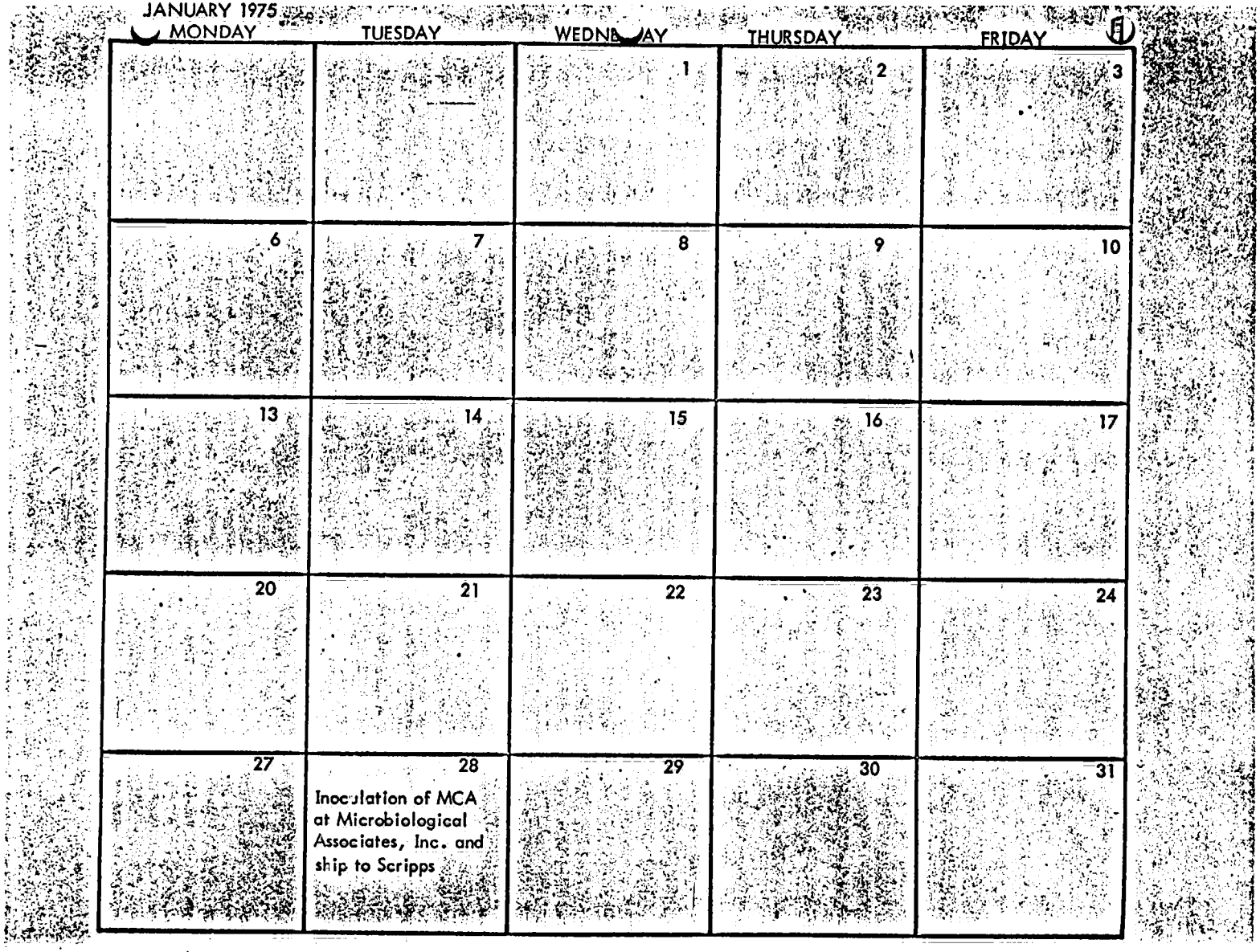
The following protocol was followed to arrive at the above conclusion. Goat erythrocytes (GRBC), with a mosaic of antigens on the surface, were selected as the test antigen. Three strains of mice were used (C_3H, $DBA/_2$, C_{57} $Bl/_6$); two are AHH inducible; one is not. Three different histocompatibility types (H-2) are represented. Either MCA in gelatin, gelatin alone, or saline was administered IT. Six days later, mice were immunized with GRBC, and ten days after that were re-immunized. Individual assays to quantitate the number of cells making antibody to GRBC were performed 3, 5, 7 or 9 days after the secondary immunization. This kinetic approach eliminated the possibility that a delay in peak response induced by MCA would be mistaken for true suppression. For each animal immunized with GRBC, an identical animal was immunized with saline; this allowed us to detect so-called "natural" immunity. Since we studied the secondary response to an antigen (two immunizations) a normal response was conclusive evidence that both thymus dependent and bone marrow dependent lymphoid populations were functional in these mice.

The results are presented on attached pages in tabular and graphic format. This study very clearly demonstrated significant immunosuppression lasting at least 19 days after IT administration of MCA in strains of mice that develop lung tumors after MCA is given IT (C_3H and C_{57} $Bl/_6$) and are inducible for the AHH enzyme system. In $DBA/_2$ mice, which are resistant to the carcinogenic effects of MCA and are AHH non-inducible, there is no immunosuppression observed. This is a very exciting result, as it presents us with an opportunity to dissect the role of immunosuppression in development of lung tumors after chemical carcinogens are administered.

APPENDED CALENDARS

FOR WORK TO BE PERFORMED

AS DESCRIBED IN SECTION 10



JANUARY 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
		1	2	3
6	7	8	9	10
13	14	15	16	17
20	21	22	23	24
27	28 Inoculation of MCA at Microbiological Associates, Inc. and ship to Scripps	29	30	31

FEBRUARY 1975

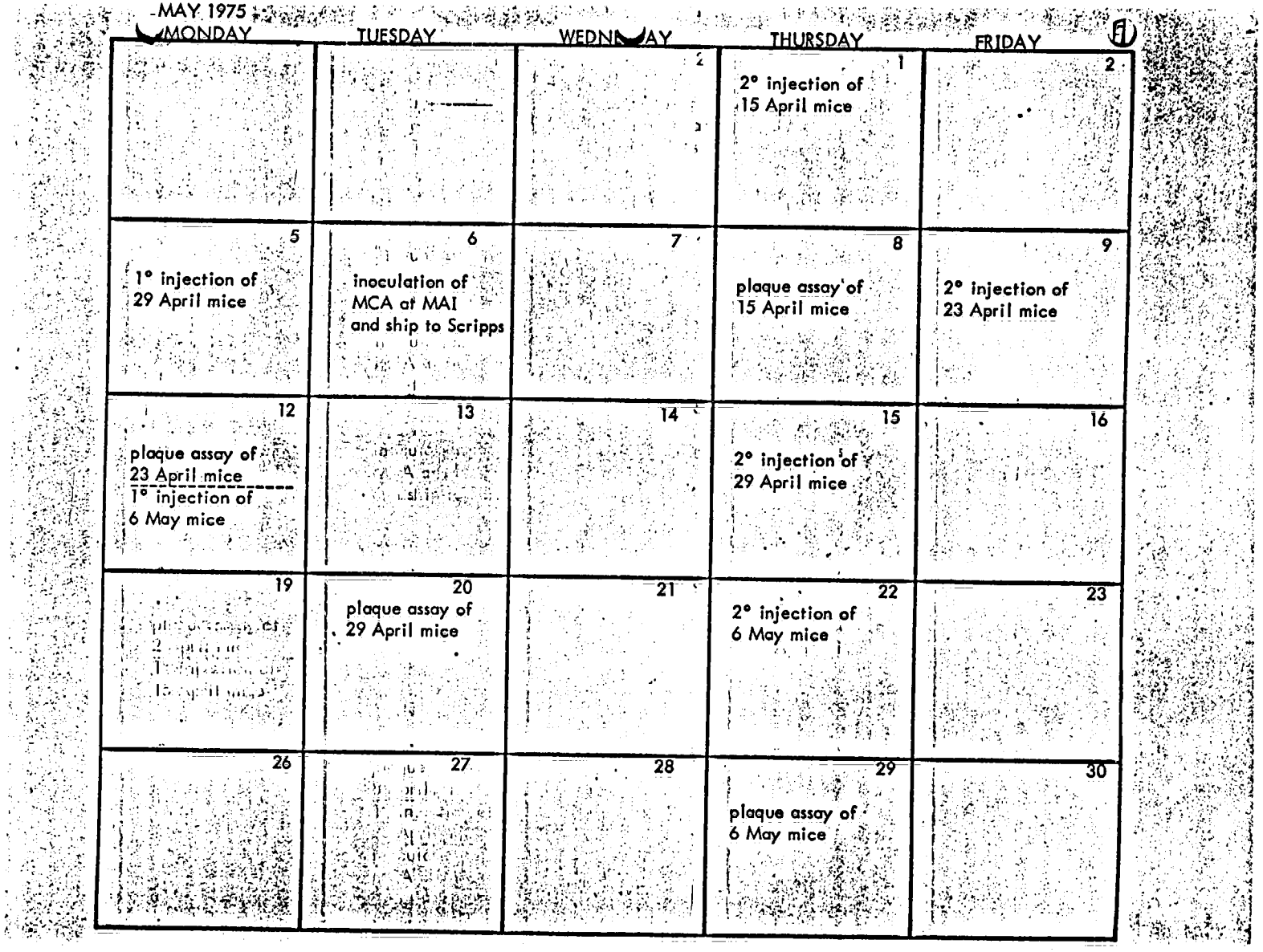
MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
3 1° injection of mice treated 28 Jan	4	5 Inoculation of MCA at MAI and ship to Scripps	6	7
10	11 1° injection of 5 Feb mice Inoculation of MCA at MAI and ship to Scripps	12	13 2° injection of 5 Feb mice	14
17 1° injection of 11 Feb mice	18 plaque assay of 28 Jan mice	19 Inoculation of MCA at MAI and ship to Scripps	20	21 2° injection of 5 Feb mice
24 plaque assay of 28 Jan mice	25 1° injection of 19 Feb mice inoculation of MCA at MAI and ship to Scripps	26	27 2° injection of 11 Feb mice	28

MARCH 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
3 1° injection of 25 Feb mice	4 inoculation of MCA at MAI and ship to Scripps	5	6 plaque assay of 11 Feb mice	7 2° injection of 19 Feb mice
10 plaque assay of 19 Feb mice 1° injection of 4 March mice	11	12 inoculation of MCA at MAI and ship to Scripps	13 2° injection of 25 Feb mice	14
17	18 plaque assay of 25 Feb mice 1° injection of 12 March mice inoculation of MCA at MAI and ship to Scripps	19	20 2° injection of 4 March mice	21
24 1° injection of 18 March mice	25 inoculation of MCA at MAI and ship to Scripps	26	27 plaque assay of 4 March mice	28 2° injection of 12 March mice
31 plaque assay of 12 March mice 1° injection of 25 March mice				

APRIL 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
1° injection of 25 [illegible] mice	1	2 inoculation of MCA at MAI and ship to Scripps	3 2° injection of 18 March mice	4
7	8 plaque assay of 18 March mice 1° injection of 2 April mice inoculation of MCA at MAI and ship to Scripps	9	10 2° injection of 25 March mice	11
14 1° injection of 8 April mice	15 inoculation of MCA at MAI and ship to Scripps	16	17 plaque assay of 25 March mice	18 2° injection of 2 April mice
21 plaque assay of 2 April mice 1° injection of 15 April mice	22	23 inoculation of MCA at MAI and ship to Scripps	24 2° injection of 8 April mice	25
28 1° injection of 25 [illegible] mice	29 plaque assay of 8 April mice 1° injection of 23 April mice inoculation of MCA at MAI and ship to Scripps	30		



MAY 1975

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
			1 2° injection of 15 April mice	2
5 1° injection of 29 April mice	6 inoculation of MCA at MAI and ship to Scripps	7	8 plaque assay of 15 April mice	9 2° injection of 23 April mice
12 plaque assay of 23 April mice 1° injection of 6 May mice	13	14	15 2° injection of 29 April mice	16
19	20 plaque assay of 29 April mice	21	22 2° injection of 6 May mice	23
26	27	28	29 plaque assay of 6 May mice	30

GRAPHIC REPRESENTATION OF DATA

OBTAINED FROM STUDY OF IMMUNE COMPETENCE

IN MCA TREATED AND CONTROL MICE

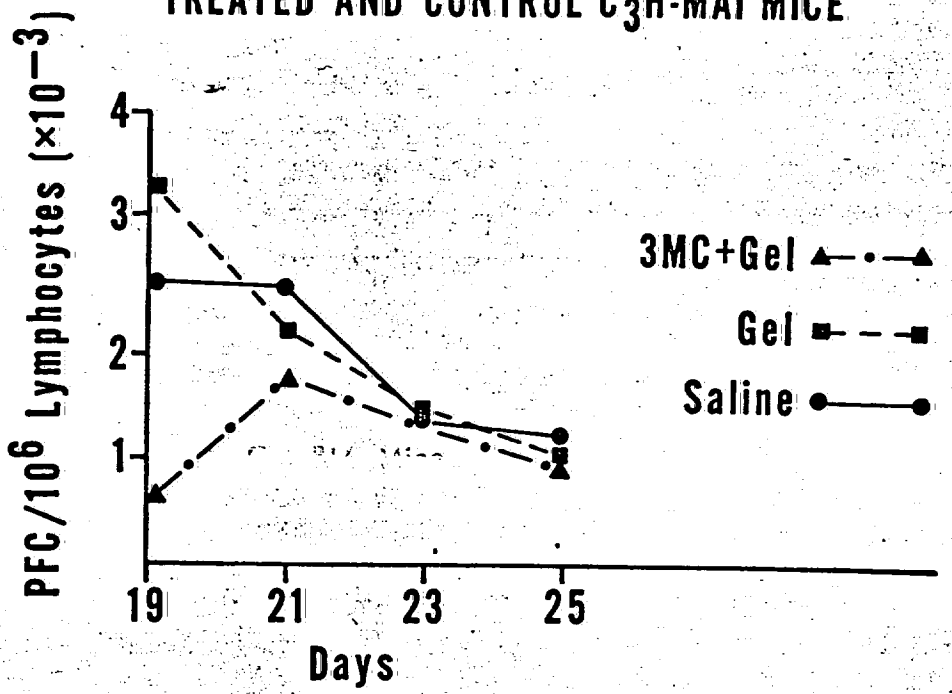
A. $C_3H/_F$ MAI Mice

B. C_{57} Bl/6 Mice

C. DBA/2 -J Mice

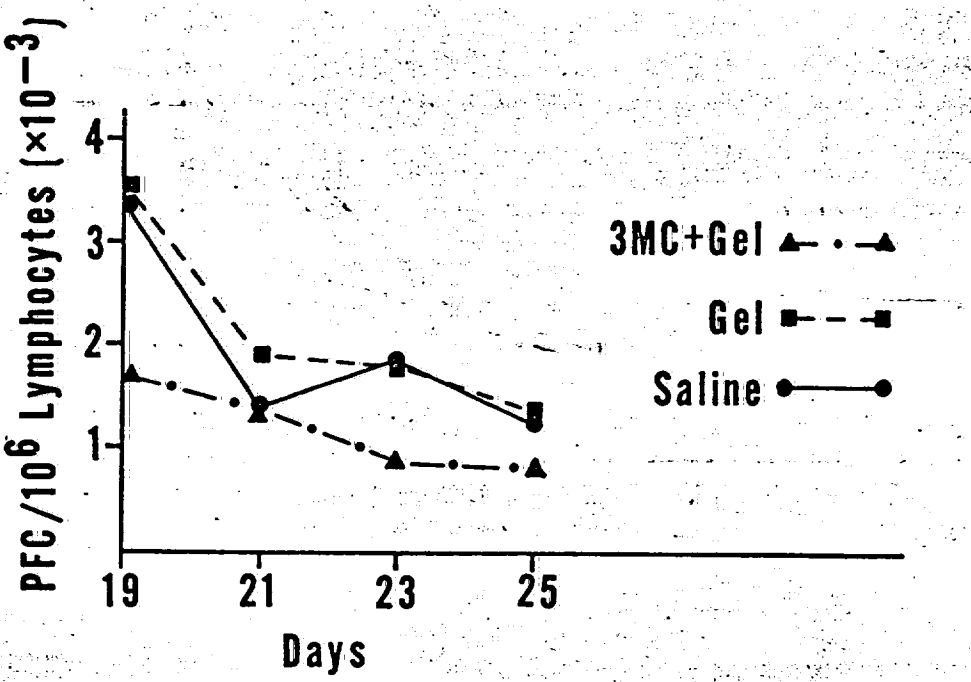
D. Composite of All Data

ANTI-GOAT ERYTHROCYTE PLAQUE FORMING CELLS (INDIRECT) IN SPLEENS OF 3-METHYL CHOLANTHRENE TREATED AND CONTROL C_3H-MAI MICE



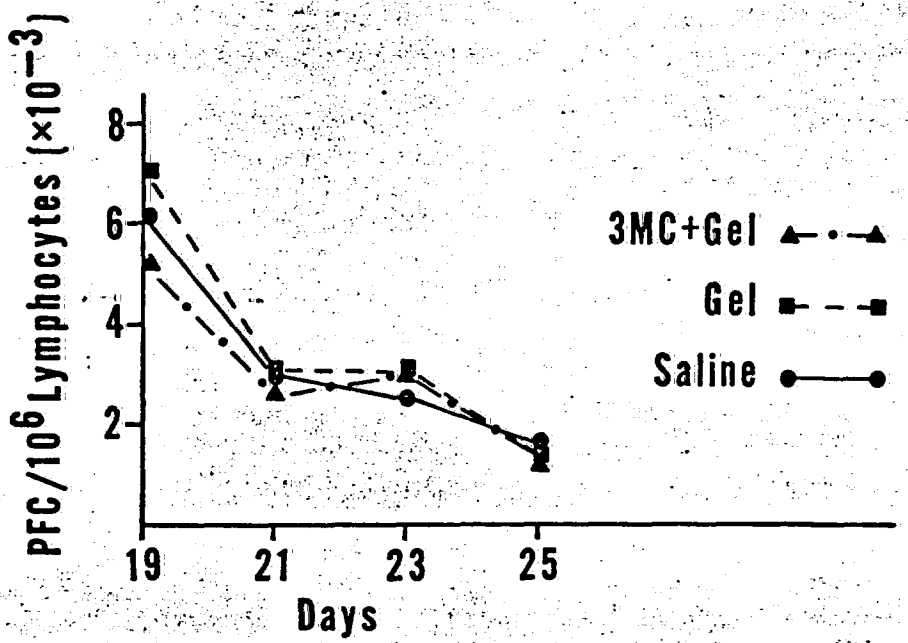
GRAPHIC REPRESENTATION - A

ANTI-GOAT ERYTHROCYTE PLAQUE FORMING CELLS (INDIRECT) IN SPLEENS OF 3-METHYL CHOLANTHRENE TREATED AND CONTROL C_{57}-BL/$_6$-Cum MICE



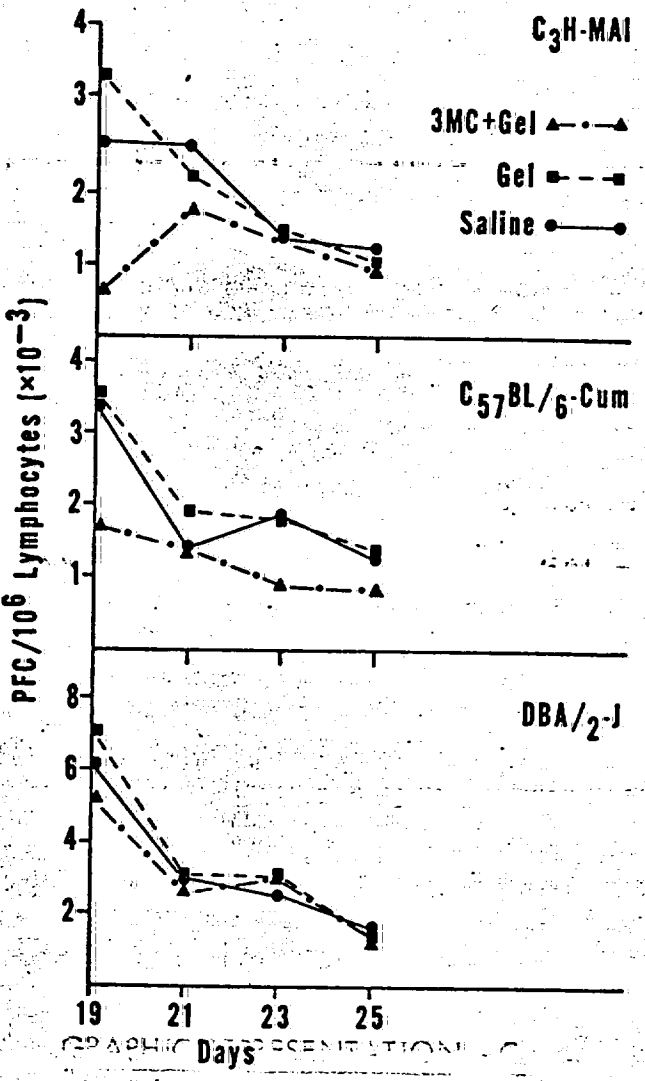
GRAPHIC REPRESENTATION - B

ANTI-GOAT ERTHROCYTE PLAQUE FORMING CELLS (INDIRECT) IN SPLEENS OF 3-METHYL CHOLANTHRENE TREATED AND CONTROL DBA/$_2$-J MICE



GRAPHIC REPRESENTATION - C

ANTI-GOAT ERYTHROCYTE PLAQUE FORMING CELLS (INDIRECT) IN SPLEENS OF 3-METHYL CHOLANTHRENE TREATED AND CONTROL MICE



GRAPHIC REPRESENTATION - D

TABULAR REPRESENTATION OF DATA

OBTAINED FROM STUDY OF IMMUNE COMPETENCE

IN MCA TREATED AND CONTROL MICE

Each number represents 10 individual assays. Background, representing "natural" immunity, has been subtracted for each assay.

A. Indirect (IgG) Plaque Forming Cells Per 10^6 spleen lymphocytes.

B. Direct (IgM) Plaque Forming Cells Per 10^6 spleen lymphocytes.

TABULAR REPRESENTATION - A

INDIRECT PFC/10^6 SPLEEN LYMPHOCYTES

		Day 13	Day 15	Day 17	Day 19
	N	344	1227	1470	1089
C_3H ♀	R	2828	2353	1553	1083
	L	2263	3157	1292	1284
	N	664	2218	899	552
C_3H ♂	R	3692	2143	1391	864
	L	2946	1944	1363	1095
	N	6310	3018	3479	1441
DBA ♀	R	6698	3681	3089	1685
	L	5157	3140	2727	1739
	N	3304	1679	2068	1302
DBA ♂	R	7152	2127	3043	1035
	L	6898	2242	2094	1423
	N	1629	1404	1165	900
$C_{57}Bl/_6$ ♀	R	3384	1890	2571	1242
	L	4364	1432	2186	1344
	N	1579	1238	555	640
$C_{57}Bl/_6$ ♂	R	3558	1785	1061	1472
	L	2422	1285	1493	1089

TABULAR REPRESENTATION - B

DIRECT PFC/10^6 SPLEEN LYMPHOCYTES

		Day 13	Day 15	Day 17	Day 19
	N	198	82	215	81
C_3H ♀	R	635	197	91	109
	L	519	172	243	171
	N	366	124	77	59
C_3H ♂	R	594	114	126	96
	L	563	216	79	102
	N	136	51	115	40
DBA ♀	R	109	76	104	77
	L	298	83	188	94
	N	93	41	27	43
DBA ♂	R	275	87	61	44
	L	423	447	58	170
	N	638	139	78	80
$C_{57}Bl/_6$ ♀	R	421	130	151	80
	L	734	101	127	71
	N	511	115	56	109
$C_{57}Bl/_6$ ♂	R	537	120	86	150
	L	575	186	124	110

10035368 39

13. Budget for the coming year:

		% Time	Amount
A.	Salaries:		
	Professional:		
	Lerner, Richard A.	15%	-0-
	Levy, Richard L.	40%	9,280
	Technical:		
	Technicians (2)	100%	20,000
	Sub-Total for A		29,280
B.	Consumable Supplies:		
	Supplies 11,000		
	Animals 10,000		
	Sub-Total for B		21,000
C.	Other Expenses:		
	Travel and Shipping Costs 3,000		
	Part time services of secretary, animal caretakers, photographer, histology technician, EM technician, electronic repairman and machinist 4,000		
	Sub-Total for C		7,000
	Running Total of A+B+C		57,280
D.	Permanent Equipment:		
	None		
E.	Indirect Costs (15% of A+B+C)		8,592
	TOTAL REQUEST		65,872